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13. ABSTRACT (Maximum 200 Words)

The aim of this project was to understand the role and the mechanism of bone morphogenetic protein-2 (BMP-2, a protein mainly known to help in bone remodeling) in breast cancer cell growth and bone metastasis. To this end we have conclusively shown that BMP-2 is a potent inhibitor of breast cancer cell growth in a nude mouse xenograft model. We have also been successful in establishing an animal model to study bone metastsis from xenograft experiments. We have recently got data from our preliminary experiments to suggest that BMP-2 has the potential to reduce osteolysis following breast cancer development in the nude mice model. For the past year during the no cost extension period of my project we tried to develop some new ideas related to our findings from this project. We looked at the mechanism of BMP-2 induced inhibition of breast cancer cell growth in the tumors formed in the presence of BMP-2. We also started to look into the role of dietary supplements to increase the growth inhibitory property of BMP-2 in this mouse model. The proposed experiments were include in a new DOD IDEA proposal this year and is pending review later this year. The preliminary experiments point to an interesting finding showing that fish oil supplement in diet of mice can effectively reduce breast cancer cell growth and BMP-2 might be involved in this mechanism.

14. SUBJECT TERMS

Human breast cancer, bone morphogenetic protein-2 (BMP-2), xenograft model, growth inhibition.

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Introduction:

As we discussed in our final report our aim was to identify novel therapeutic application for BMP-2 in breast cancer cell growth inhibition. We have shown that in *in vitro* cell culture system BMP-2 inhibits growth of both estrogen-dependent (MCF-7) and estrogen-independent (MDA MB 231) human breast cancer cells (1, 2). We also elucidated the underlying mechanism for BMP-2-induced breast cancer cell growth inhibition during the project period. We have shown the involvement of cell cycle regulatory proteins cyclin D1, cyclin dependent kinases and cell cycle inhibitor p21 in BMP-2-mediated breast cancer cell growth inhibition (1). In addition, the activity of the tumor suppressor protein, retinoblastoma (pRb) was also increased by BMP-2 in these human breast cancer cells (1). During last year of this project period we elucidated the effect of BMP-2 on a new tumor suppressor protein, PTEN (phosphatase and tensin homolog deleted from chromosome 10), in human breast cancer cells. We have also continued to elucidate the role of BMP-2 in bone metastasis of breast cancer cells in a nude mouse model that we developed during this project period. The following is a description of our experiments for this final addendum period of this proposal.

Body:

Task 1. **To correlate the levels of BMP-2 and BMP-2 receptor expression in breast cancer cell lines and tissue samples with their bone metastasis status (months 1-12)**

- **Perform Northern as well as the RT-PCR analysis of BMP-2 and BMPR expression in different ER negative and ER positive cells and in different breast tumor tissue samples (months 1-6).**

  Completed.

- **Analyze the expression level of BMP-2 and BMPR different ER negative and ER positive cells and in different breast tumor tissue samples (months 6-7).**

  Repository cannot be accessed as explained in Final Report (2002)

- **Correlate the metastatic potential of the breast cancer cells using the animal model described above *by in vivo* study (months 6-12).**

  We have developed a nude mouse model where the bone metastasis can be followed in a xenograft experiment.

- **Correlate metastatic property of the tissue samples with the expression levels of BMP-2 and BMPR in these samples (months 7-8).**

Repository cannot be accessed as explained in Final Report (2002)

Task 2. **To prove the clinical importance of BMP-2 expression and bone metastasis of breast cancer cells by genetically engineering the BMP-2 status of breast cancer cells and study the metastatic phenotype of the altered cells using an *in vivo* model of metastasis (months 6-24).**

Last year in my report I showed data that the adenovirus vector expressing BMP-2 (AdBMP-2) can effectively inhibit breast cancer cell growth in a nude mouse model. We have used the same vector to test the effect of BMP-2 on bone metastasis of breast cancer cells. Bone metastasis is measured by the degree of osteolysis which is detected by X-Ray analysis of the bones from mice injected with human breast cancer cells. We have injected AdBMP-2-infected MCF-7 cells in nude mice (the mice were given estradiol 3 days prior to MCF7 injection) and followed the osteolysis of their bone. The amount of osteolysis is directly proportionate with the extent of bone metastasis in these mice. The results from our preliminary experiments show that the BMP-2 produced by the AdBMP-2 was sufficient to decrease the amount of osteolysis (Fig 1). We are currently repeating this study to get statistically significant data.

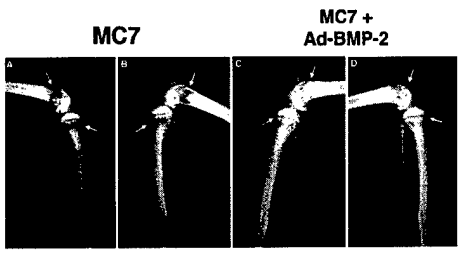


Fig 1. Inhibition of osteolysis by BMP-2 expressiong adenoviral vector (AdBMP-2). X-Ray analysis of long bones of nude mice injected with uninfected (Panels A, B) or AdBMP-2 infected (Panels C, D) MCF-7 cells. Areas of osteolysis are indicated by arrows.

Task 3. **To study differential effect of BMP-2 on ER-positive and ER-negative breast cancer cell growth and to investigate the underlying mechanism (months 6-18).**

- **Study effect of BMP-2 on ER-negative breast cancer cells *in vitro* by Flowcytometric analysis of cell cycle progression (months 6-8).**

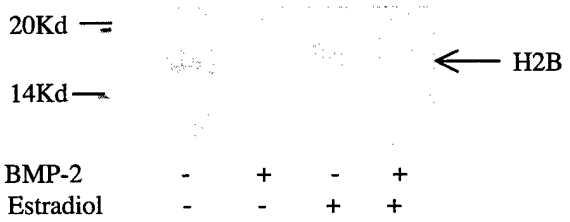
Study the underlying mechanism of BMP-2 induced growth regulation of breast cancer cells. This part will include determination of the effect of BMP-2 on the MAPK pathway by enzymatic activity assay for MAPK, studying growth kinetics of breast cancer cells in the presence of MEK inhibitor and identification of other targets for BMP-2 action in these cells (months 9-24).

Completed (see reports from 2000 and 2001) (1, 2).

Additional study for underlying mechanism of BMP-2 action:

We have recently reported a novel signaling pathway for BMP-2 in osteoblast cell differentiation (3, 4). BMP-2 is a growth and differentiation promoting factor for the osteoblast cells. We have shown that BMP-2 increase Akt kinase activity in osteoblast cells. In contrast BMP-2 is a growth inhibitory protein for the breast cancer cells. Thus we measured Akt kinase activity in MCF-7 human breast cancer cells treated with BMP-2 in the presence or absence of estradiol. The result shows that in contrast to the effect of BMP-2 in osteoblast cells, BMP-2 inhibits Akt kinase

BMP-2 decreases Akt activity in MCF7 cells



Fifg 2. Akt kinase assay was performed as described before in our publications (Appendix 3) using H2B as a substrate. Pphosphorylated H2B was indicated by arrow.

BMP-2 increases PTEN expression in MDA MB 231 cells

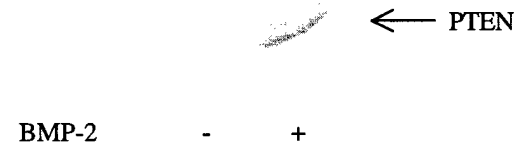


Fig 3. Western analysis ofBMP-2-treated MDA MB 231 cell lysates for PTEN expression.

activity in breast cancer cells in the presence or absence of estradiol (Fig 2). Akt kinase activity is necessary for cell growth. Thus inhibition of Akt kinase activity could be another mechanism of growth inhibition of breast cancer cells by BMP-2. We will be extending on this part of the mechanism in future.

Since we found that BMP-2 increases the activity of the tumor suppressor protein pRB in breast cancer cells (1) we have tested the effect of BMP-2 on other tumor suppressor proteins. We found that BMP-2 significantly increases the protein level of the tumor suppressor protein PTEN in MDA MB 231 cells (Fig 3). PTEN tumor suppressor protein inhibits the activity of PI 3 kinase, a potent growth promoting signaling molecule. Activation of PI 3 kinase in turn activates Akt kinase in growing cells. Thus BMP-2 by increasing PTEN protein level may inhibit PI 3 kinase activity in breast cancer cells and thus can reduce Akt kinase activity as reported above (Fig 2). We will be further testing this signaling pathway in breast cancer cells.

Task 4. **To correlate the growth inhibitory effect of BMP-2 on ER-positive breast cancer cells *in vivo* (months 18-36).**

- **To study autocrine BMP-2 action (*in vitro*) : Analysis of effect of estradiol on growth kinetics of MCF-7 cells stably transfected with BMP-2 cDNA (months 18-24).**

Not continued as explained in Final Report of 2002.

- **To study autocrine BMP-2 action (*in vivo*) : Xenograft tumor formation assay of MCF-7 cells stably transfected with BMP-2 cDNA or vector alone (months 24-36).**

Still underway.

Key Research Accomplishments:

- Identification of BMP-2 as a potential in vitro growth inhibitor for the breast cancer cells irrespective of their estrogen responsiveness.
- Understanding of the underlying mechanism of BMP-2-induced inhibition of breast cancer cell growth in vitro.
- Generation of *in vivo* mouse model for studying bone metastasis of breast cancer cells.
- *In vivo* confirmation of breast cancer growth inhibitory properties of BMP-2.
- *In vivo* confirmation of inhibition of skeletal metastasis of breast cancer cells by BMP-2.

Reportable Outcome:

Abstracts:

1. BMP-2 inhibits EGF-induced MDA-MB231 breast cancer cell growth via increased expression of p21 WAF1 cyclin kinase inhibitor (CKI) by posttranscriptional mechanism. N. Ghosh-Choudhury, S.L. Abboud and G. Ghosh-Choudhury. American Society of Bone and Mineral Research 21st Annual meeting, 1999. **(Poster Presentation)**
2. Mechanism for inhibitory role of BMP-2 on growth of estrogen-responsive and non-responsive human breast cancer cells. N. Ghosh-Choudhury, S.L. Abboud and G. Ghosh-

Choudhury. International Conference Bone Morphogenetic Proteins 2000. **(Oral Presentation)**

Manuscripts:

1. Ghosh-Choudhury, N., Ghosh-Choudhury, G., Celeste, A., Ghosh, P. M., Moyer, M., Abboud, S. L., and Kreisberg, J. Bone morphogenetic protein-2 induces cyclin kinase inhibitor p21 and hypophosphorylation of retinoblastoma protein in estradiol-treated MCF- 7 human breast cancer cells. Biochim Biophys Acta, *1497:* 186-196., 2000.
2. Ghosh-Choudhury, N., Woodruff, K., Qi, W., Celeste, A., Abboud, S. L., and Ghosh Choudhury, G. Bone morphogenetic protein-2 blocks MDA MB 231 human breast cancer cell proliferation by inhibiting cyclin-dependent kinase-mediated retinoblastoma protein phosphorylation. Biochem Biophys Res Commun, *272:* 705-711., 2000.
3. Ghosh-Choudhury, N., Abboud, S. L., Nishimura, R., Celeste, A., Mahimainathan, L., and Choudhury, G. G. Requirement of BMP-2-induced phosphatidylinositol 3-kinase and Akt serine/threonine kinase in osteoblast differentiation and Smad- dependent BMP-2 gene transcription. J Biol Chem, *277:* 33361-33368., 2002.
4. Ghosh-Choudhury, N., Sherry, A. L., Mahimainathan, L., Chandrasekar, B., and Ghosh Choudhury, G. Phosphatidylinositol 3 kinase regulates BMP-2-induced MEF-2A-dependent transcription of BMP-2 gene in cardiomyocyte precursor cells. J Biol Chem, 2003.

Conclusions:

Four years ago we had started analyzing the role of a novel protein in the breast cancer cell growth mechanism. This protein, known as bone morphogenetic protein-2 (BMP-2) has been characterized before as a modulator of bone cell growth. We show that BMP-2 can significantly inhibit the growth of breast cancer cells *in vitro*. We have studied this growth inhibitory role of BMP-2 in estrogen-responsive and non-responsive breast cancer cells and have found that it inhibits the growth of both the cell types with equal potency. In estradiol-responsive human breast cancer cells, BMP-2 can inhibit estradiol-induced growth of these cells. We have also identified the mechanism by which BMP-2 inhibits the growth of these cells (1, 2) . Recently we have developed *in vivo* model system to test this *in vitro* phenomenon. We have found that BMP-2 inhibits breast tumor growth in this mouse model significantly. We have also provided evidence in this report that BMP-2 might also block bone metastasis of breast cancer cells in this *in vivo* model. If BMP-2 is found out to be statistically effective *in vivo*, then BMP-2 will prove to be a clinically important molecule for the breast cancer patients.

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1. Ghosh-Choudhury, N., Ghosh-Choudhury, G., Celeste, A., Ghosh, P. M., Moyer, M., Abboud, S. L., and Kreisberg, J. Bone morphogenetic protein-2 induces cyclin kinase inhibitor p21 and hypophosphorylation of retinoblastoma protein in estradiol-treated MCF- 7 human breast cancer cells. Biochim Biophys Acta, *1497:* 186-196., 2000.
2. Ghosh-Choudhury, N., Woodruff, K., Qi, W., Celeste, A., Abboud, S. L., and Ghosh Choudhury, G. Bone morphogenetic protein-2 blocks MDA MB 231 human breast cancer cell proliferation by inhibiting cyclin-dependent kinase-mediated retinoblastoma protein phosphorylation. Biochem Biophys Res Commun, *272:* 705-711., 2000.
3. Ghosh-Choudhury, N., Abboud, S. L., Nishimura, R., Celeste, A., Mahimainathan, L., and Choudhury, G. G. Requirement of BMP-2-induced phosphatidylinositol 3-kinase and Akt

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APPENDIX 1

Bone Morphogenetic Protein-2 Blocks MDA MB 231 Human Breast Cancer Cell Proliferation by Inhibiting Cyclin-Dependent Kinase-Mediated Retinoblastoma Protein Phosphorylation

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Received May 3, 2000

Bone morphogenetic protein-2 (BMP-2) has been shown to act as an antiproliferative agent for a number of different cell types. We show that BMP-2 dose-dependently inhibits growth of MDA MB 231 human breast cancer cells. Epidermal growth factor (EGF) stimulates DNA synthesis and entry of these cells into the S-phase. BMP-2 inhibits EGF-induced DNA synthesis by arresting them in G1 phase of the cell cycle. BMP-2 increases the level of cyclin kinase inhibitor p21. Furthermore, we show that exposure of MDA MB 231 cells to BMP-2 stimulates association of p21 with cyclin D1 and with cyclin E resulting in the inhibition of their associated kinase activities. Finally, BMP-2 treatment is found to cause hypophosphorylation of the retinoblastoma protein (pRb), a key regulator of cell cycle progression. Our data provide a mechanism for the antiproliferative effect of BMP-2 in the breast cancer cells.

Key Words: **BMP-2; pRb; p21; breast cancer cells.**

The growth of breast cancer cells is regulated by a variety of steroid hormones and growth factors. In the late stage of hormone-dependent tumor cell growth, the cells become hormone-independent. One such human breast tumor cell line, MDA MB 231 (MDA), derived from a metastasized human adenocarcinoma, is estrogen-independent (1). However, they maintain the responsiveness to growth factors, such as epidermal growth factor (EGF) (2). The proliferative signals generated in the cytosol integrate into the nucleus to activate the cyclins and cyclin-dependent kinases (CDKs) that regulate the cell cycle progression of breast tumor cells (3). Thus in the early G1 phase of cell cycle, the D type cyclin activates the CDK 4 while in the mid and late G1, cyclin E and CDK2 are activated. These kinases also remain activated during S-phase of cell cycle (4, 5). These kinases phosphorylate the retinoblastoma tumor suppressor retinoblastoma protein (pRb) to drive the cells through the cell cycle (6). However, the cyclin kinase inhibitor proteins, such as p21, interacts with the cyclins thus inhibiting the CDKs resulting in blockage of cell cycle (7–9). Many agents that inhibit cell proliferation regulate the expression and activity of this group of proteins (7).

Bone morphogenetic proteins are structurally similar to the TGF β super family (10). Bone morphogenetic protein-2 (BMP-2), a member of this large family of proteins, stimulates growth and differentiation of osteogenic and chondrogenic cells during bone remodeling and also plays an important role in embryogenesis (10–13). Similar to TGF β, BMPs exert their effect via specific type I and type II serine-threonine kinase receptors. Binding of BMP-2 to the type II receptor induces the oligomerization of the receptor complex resulting in phosphorylation of the type I receptor and recruitment of downstream signaling proteins Smad 1, Smad 5 and Smad 8 (14, 15). Among these, Smad 1 has been extensively studied as the target of BMPR signaling. Type I BMPR-phosphorylated Smad1 then heterodimerizes with Smad 4 and translocates to the nucleus to act as a transcription factor and induce genes that mediate the biological activity of BMP-2 (16).

BMP-2 has recently been reported to have a growth inhibitory effect on prostate cancer cells (17). BMP-2 also inhibits smooth muscle cell proliferation (18). We have recently shown that BMP-2 inhibits PDGF and EGF-induced DNA synthesis in primary glomerular mesangial cells by inhibiting mitogen activated protein

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0006-291X/00 $35.00

kinase (MAPK) cascade (19, 20). A very high dose of BMP-2 inhibits soft agar growth of a variety of tumor samples including breast tumor (21). However the mechanism of inhibition of tumor cell proliferation by BMP-2 is not yet clear.

In this study we demonstrate that BMP-2 inhibits growth of MDA MB 231 human breast cancer cells in culture in the presence and in the absence of EGF. BMP-2 increases p21 cyclin kinase inhibitor in these cells and inhibits cyclin D1- and E-associated kinases. Furthermore BMP-2 inhibits pRb phosphorylation, which results in accumulation of cells in the G1 phase of cell cycle resulting in inhibition of DNA synthesis.

MATERIALS AND METHODS

Materials. Tissue culture reagents were purchased from Gibco/BRL (Rockville, MD). EGF was from R & D (Minneapolis, MN). Phospho pRb antibody was purchased from New England Biolabs. All other antibodies, GST-pRB and Protein A/G plus were obtained from Santa Cruz. Protein A–Sepharose CL 4B was purchased from Pharmacia. Histone H1 was purchased from Sigma. ECL reagent was purchased from Pierce laboratories. Recombinant BMP-2 was obtained from Genetics Institute.

Cell culture. MDA MB 231 cells were grown in IMEM with 5% fetal bovine serum. For experiments the cells were grown in complete medium for 48 h and serum deprived for 24 h before addition of 100 ng/ml EGF. EGF causes modest proliferative response in this isolate of MDA MB 231 cells. For cell cycle analysis, near confluent cells were used for 24 h serum-deprivation to arrest in G0/G1 phase before addition of EGF to release them. To detect the effect of EGF on MDA MB 231 cell proliferation, serum free medium was changed every 6 h.

Measurement of DNA synthesis. DNA synthesis was measured as ^{3}H-thymidine incorporation into trichloroacetic acid insoluble material as described previously (19, 20).

Flow cytometric analysis. Trypsinized MDA MB 231 cells were washed with PBS and fixed in 70% ethanol for 30 min at −20°C. The cells were then centrifuged at 1500 × g for 4 min, washed with PBS containing 1% BSA and resuspended in 150 μl PBS. For nuclear staining, the cells were treated with 50 μl of 1 mg/ml RNase A followed by 100 μl of 100 μg/ml propidium iodide and incubated at 4°C for 24 h. The cells were then analyzed by flow cytometry on FACStar Plus (Becton Dickinson Immunocytometry Systems, San Jose, CA) using 200 mW of light at 488 nm produced by an argon-ion laser. The fluorescence was read using a 630/22 nm band-pass filter. Data were analyzed for 20,000 viable cells as determined by forward and right angle light scatter and were stored as frequency histograms and subsequently analyzed by MODFIT software (Verity, Topsham, ME).

Immunoprecipitation and immunoblotting. The cells were lysed in RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1% NP-40, 1 mM PMSF and 0.1% aprotinin) for 30 min at 4°C. The cleared cell lysate was immunoprecipitated with required antibody essentially as described previously (19, 20). Immunoblotting of the immunoprecipitates or the cell lysate was also performed as described previously (19, 20, 22).

Cyclin D1- and E-associated kinase activity. The assay was performed according to the method of Gong et al (23). Briefly, cell lysates were immunoprecipitated with cyclin D1 or cyclin E antibody. The immunebeads were resuspended in kinase assay buffer (20 mM Tris-HCl, pH 7.5 and 4 mM $MgCl_2$). For cyclin D1-associated kinase assay a fragment of pRb containing the *in vivo* phosphorylation sites was used as substrate. For cyclin E-associated kinase activity, histone H1 was used as substrate. The reaction was carried out with 25 μM ATP containing 10 μCi of [γ-^{32}P]ATP for 30 min at 30°C. The reaction product was separated by SDS–polyacrylamide gel electrophoresis and the phosphorylated proteins were visualized by autoradiography.

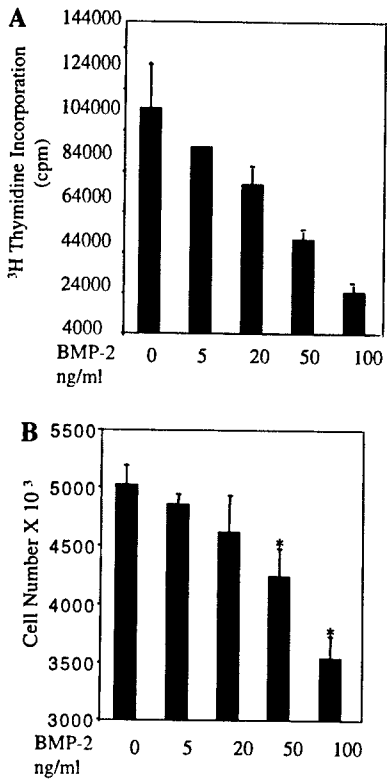

FIG. 1. (A) Effect of BMP-2 on DNA synthesis in MDA MB 231 cells. Serum-deprived MDA MB 231 cells were incubated with different concentrations of BMP-2 for 24 h. [^{3}H]Thymidine incorporation was determined as a measure of DNA synthesis, as described under Materials and Methods (19, 20). (B) Effect of BMP-2 on MDA MB 231 cell proliferation. Subconfluent cultures of MDA MB 231 cells in triplicate dishes were incubated with different concentrations of BMP-2 for 24 h. Number of cells were counted for each treatment condition. Mean ± SE of three independent experiments. *$P < 0.05$ vs untreated cells.

RESULTS

BMP-2 inhibits MDA MB 231 cell proliferation. MDA MB 231 cells grow very aggressively in culture. BMP-2 has recently been shown to have an antiproliferative effect on certain cell lines, including primary mesangial cells and prostate cancer cells (17, 19, 20). We examined the effect of BMP-2 on DNA synthesis in MDA MB 231 cells. MDA MB 231 cells were incubated with increasing concentrations of BMP-2 for 24 h. [^{3}H]Thymidine incorporation was determined as a measure of DNA synthesis in these cells. Figure 1A shows that BMP-2 inhibits DNA synthesis in a dose-dependent manner. Approximately 75% inhibition in DNA synthesis was observed at 100 ng/ml BMP-2. To

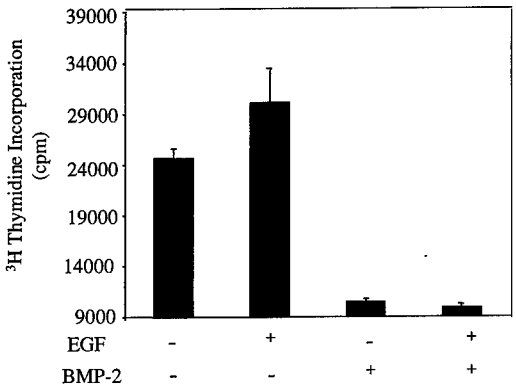

FIG. 2. Effect of BMP-2 on EGF-induced DNA synthesis. Serum-deprived MDA MB 231 cells were incubated with 100 ng/ml BMP-2 for 30 min followed by 100 ng/ml EGF for 24 h. [^{3}H]Thymidine incorporation was determined as a measure of DNA synthesis, as described under Materials and Methods (19, 20).

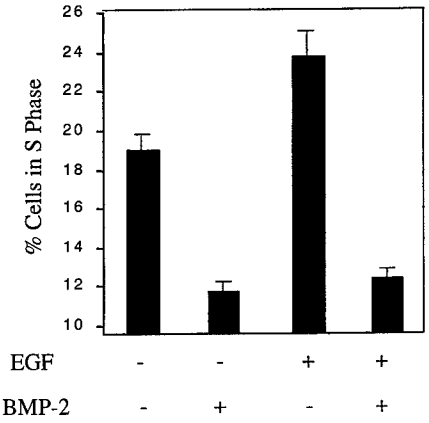

FIG. 3. S-phase analysis of EGF-induced MDA MB 231 cells. Serum-deprived MDA MB 231 cells were incubated with BMP-2 for 30 min and treated in the presence or absence of 100 ng/ml EGF for 24 h. Cells were then trypsinized and analyzed by flow cytometry as described under Materials and Methods. The percentage of cells in S-phase was plotted. Means of triplicate determinations are shown.

confirm this observed effect of BMP-2, we studied the effect of BMP-2 on MDA MB 231 cell proliferation in culture. Growing MDA MB 231 cells were treated with increasing concentrations of BMP-2. The cell number was determined. The data show that BMP-2 dose dependently inhibits MDA MB 231 cell growth in 24 h (Fig. 1B). As evident, 100 ng/ml BMP-2 inhibited proliferation of MDA MB 231 cells by 70% as compared to the untreated control. These data indicate that BMP-2 dose-dependently inhibits proliferation of MDA-MB-231 cells.

Inhibition of EGF-induced growth of MDA MB 231 cells by BMP-2. Activation of EGF receptor family is often associated with breast cancer cell growth (24). To test the effect of BMP-2 on EGF-induced DNA synthesis in MDA MB 231 cells, [^{3}H]thymidine incorporation was determined in the presence and absence of BMP-2. As shown in Fig. 2, EGF increases the DNA synthesis in MDA MB 231 cells by 20%. However, BMP-2 inhibits DNA synthesis by 58% in these cells in the presence of EGF. Since the increase in DNA synthesis by EGF was modest, we tested the effect of EGF on MDA MB 231 cell cycle progression using FACS analysis. Using this technique, EGF increased the number of cells in S phase by 31% (Fig. 3). These data indicate that the mitogenic effect of EGF on MDA MB 231 cells is due to increased progression of these cells from G1 to S phase. Incubation with BMP-2 shows a 58% decrease of cells in the S-phase (Fig. 3). This decrease in the S phase population is accompanied by an increase in cell number at the G1 phase of cell cycle (Table 1). These data indicate that BMP-2 inhibits EGF-induced MDA MB 231 cell proliferation by arresting them at the G1 phase of cell cycle.

BMP-2 induces CDK inhibitor p21 in MDA MB 231 cells. The key proteins that regulate cell cycle progression from G1 to S phase are cyclins D and E and the CDKs that are associated with these cyclins (4, 5). The presence of cyclin kinase inhibitor such as p21 causes cell cycle arrest in G1 by quenching cyclin D and E resulting in inhibition of their associated kinase activity (7–9). Since BMP-2 inhibits G1 to S phase transition (Fig. 3), we tested the effect of BMP-2 on p21 expression in the presence and absence of EGF by immunoblot analysis. As shown in Fig. 4, EGF does not have any significant effect on p21 protein level. However, BMP-2 increases the abundance of p21 in these cells in the presence (compare lane 4 with 3) and in the absence (compare lane 2 with 1) of EGF. These data provide the first evidence that BMP-2 may inhibit MDA MB 231 cell proliferation by increasing the level of p21.

Increased association of p21 with cyclin D1 and cyclin E in the presence of BMP-2. Activation of CDKs is an important step for cell cycle progression from G1 to S phase (3). p21, by associating with cyclins D1 and E, makes them unavailable for activating CDKs and thus

TABLE 1

Flow Cytometric Analysis of MDA MB 231 Cells for G1 and S Phase Quantitation

	% of cells in G1 phase	% of cells in S phase
Control	40.86	19.40
EGF	35.51	24.15
BMP-2	45.32	12.10
EGF + BMP-2	42.93	12.87

Note. Serum-starved MDA MB 231 cells were treated with 100 ng/ml EGF in the presence and absence of 100 ng/ml BMP-2. The cells were analyzed by flow cytometric technique for quantitation of cells present in G1 and S phase.

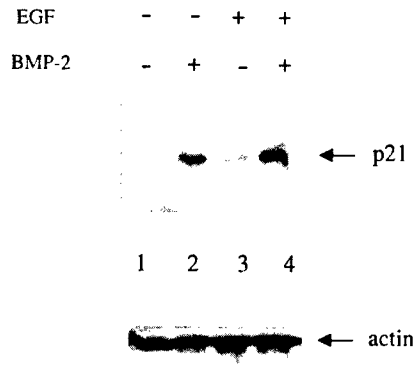

FIG. 4. Effect of BMP-2 on the cyclin kinase inhibitor p21 expression. Serum-deprived MDA MB 231 cells were incubated with EGF in the presence and absence of BMP-2. Equal amounts of cell lysates were analyzed by immunoblotting with p21 antibody and the signal was developed by ECL as described under Materials and Methods. Arrow indicates the position of p21 protein (upper panel). Lower panel shows immunoblotting of same samples with anti-actin antibody.

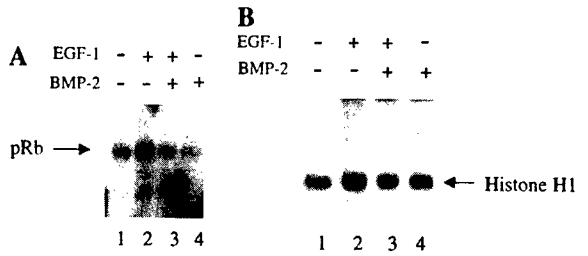

FIG. 6. Effect of BMP-2 on EGF-induced cyclin D1- and cyclin E-associated kinase activity. Serum-deprived MDA MB 231 cells were incubated with EGF in the presence or absence of BMP-2. The lysates were immunoprecipitated with cyclin D1 (A) or cyclin E (B) antibodies. The immunoprecipitates were assayed for kinase activity in the presence of [γ^{32}P]ATP using pRb (A) or histone H1 (B) as substrates. The labeled protein was separated by SDS gel electrophoresis and visualized by autoradiography. The arrow indicates the phosphorylated pRb (A) and histone H1 (B).

inhibits cell cycle progression to S phase (7–9). Since BMP-2 increased the level of p21 (Fig. 4), we investigated whether p21 associates with cyclin D1 and cyclin E in BMP-2-treated MDA MB 231 cells in the presence or absence of EGF. p21 was immunoprecipitated from cells treated with EGF and combination of BMP-2 and EGF. The immunoprecipitates were immunoblotted either with cyclin D1 (Fig. 5A) or with cyclin E (Fig. 5B) antibody. The results show that EGF does not have any significant effect on association of p21 with these two cyclins. In contrast, incubation of cells with BMP-2 results in increased association of p21 with cyclin D1 and cyclin E in the presence (compare lanes 3 with 1 in Figs. 5A and 5B) or in the absence (compare lanes 2 with 4) of EGF. These data indicate that increased association of p21 with cyclin D1 and E may inhibit the kinase activities associated with these cyclins.

BMP-2 treatment inhibits kinase activity associated with cyclin D1 and cyclin E. Cell cycle progression depends on phosphorylation of a number of regulatory

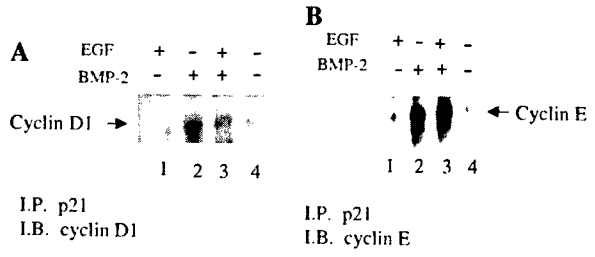

FIG. 5. Effect of BMP-2 on association of p21 with cyclin D1 and cyclin E. Equal amounts of cleared cell lysates from MDA MB 231 cells, treated with EGF in the presence or absence of BMP-2, were immunoprecipitated (I.P.) with anti-p21 antibody. The immunoprecipitates were analyzed by immunoblotting (I.B.) with anti-cyclin D1 antibody (A) and anti-cyclin E antibody (B). The arrows indicate the positions of cyclin D1 and cyclin E in A and B, respectively.

proteins by CDKs. In the G1 phase of cell cycle, cyclin D1-associated kinase is activated to initiate the cells to progress into S phase (4, 5). Since BMP-2 increases the association of cyclin kinase inhibitor p21 with cyclin D1 (Fig. 5A) and inhibits G1 to S phase progression in MDA MB 231 cells (Fig. 3), we investigated the effect of BMP-2 on cyclin D1-associated kinase activity. The lysates of MDA MB 231 cells treated with either EGF or EGF plus BMP-2 were immunoprecipitated with cyclin D1 antibody and assayed for associated kinase activity using pRb as substrate. As shown in Fig. 6A, EGF increases the pRb phosphorylation by cyclin D1-associated kinase (compare lane 2 with lane 1). BMP-2 inhibits EGF-induced pRb phosphorylation (compare lane 4 with lane 2). In mid G1 to S phase of cell cycle, cyclin E-associated kinase is activated (5, 25). Therefore, we immunoprecipitated cyclin E from lysates of MDA MB 231 cells and assayed for associated kinase activity using histone H1 as substrate. Figure 6B shows that EGF stimulates cyclin E-associated kinase activity (compare lane 2 with lane 1). However, BMP-2 inhibits EGF-induced histone H1 kinase activity (compare lane 4 with lane 2). During cell cycle progression, CDK2 remains activated in the S phase. Immunecomplex kinase assay of CDK2 immunoprecipitates showed increased CDK2 activity by EGF and BMP-2 inhibited EGF-induced CDK2 activity (data not shown). These data indicate that BMP-2 intercepts cyclin-dependent kinases to inhibit cell cycle progression of MDA MB 231 cells.

BMP-2 blocks pRb phosphorylation in MDA MB 231 cells. Activation of CDKs during mid G1 and S phase of cell cycle phosphorylate the retinoblastoma tumor suppressor protein pRb to drive the cells through cell cycle (6, 7). In MDA MB 231 cells the level of pRb phosphorylation was determined using a phospho-pRb antibody. An immunoblot analysis of lysates of cells treated with EGF alone and EGF plus BMP-2 is shown in Fig. 7. Lysates from control and

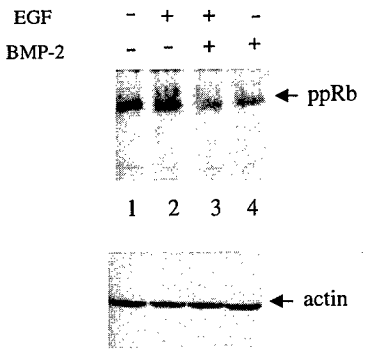

FIG. 7. Effect of BMP-2 on EGF-induced pRb phosphorylation. 50 μg cleared cell lysates of MDA MB 231 cells, treated with EGF in the presence or absence of BMP-2, were immunoblotted with an anti-phospho-pRb antibody. The arrow indicates phosphorylated pRb (ppRb).

EGF-treated cells show increased pRb phosphorylation (lanes 1 and 2). But in cells treated with BMP-2, the level of total phosphorylated pRb is significantly reduced (lane 3 and 4). One reason of increased phosphorylation in control MDA MB 231 cells may be due to their aggressive growth even in the absence of serum. These data provide the first evidence that BMP-2 inhibits pRb phosphorylation and indicate that this inhibition of pRb phosphorylation by cyclin-dependent kinases may result in attenuation of MDA MB 231 cell proliferation.

DISCUSSION

Our data in this study provide the first evidence that BMP-2 inhibits EGF-induced proliferation of MDA MB 231 cells by blocking cyclin dependent kinase activities and pRb phosphorylation. This inhibition may be due to BMP-2-induced increase in p21 cyclin kinase inhibitor level in these cells.

Breast cancer cells often metastasize to bone presumably because of a favorable growth-promoting environment provided by the bone where various growth factors are expressed in abundance (26). Our data show that one of the potent osteogenic factors, BMP-2, inhibits proliferation of MDA MB 231 cells in culture (Fig. 1). This observation was unexpected, as it was logical to predict that BMP-2, which is present in the microenvironment of bone, may not have any effect, or it may support growth of these cancer cells present in the microenvironment of bone. However, BMP-2 is present in bone matrix at a very low concentration (1–2 ng/g), whereas other growth factors such as insulin-like growth factor II (IGFII) is present at a concentration of 1500 ng/g (26, 27). Thus it is possible that the concentration of BMP-2 in the bone microenvironment may not be sufficient to inhibit the breast cancer cell growth. This may explain the requirement of a high concentration of BMP-2 to inhibit the proliferation of MDA MB 231 cells in culture (Fig. 1). Also we have shown previously that BMP-2 inhibits proliferation of primary mesangial cells at a very high concentration (19, 20). It should be noted that inhibition of prostate cancer cell proliferation is also achieved at a relatively high concentration (17).

EGF receptors in many breast cancer cells play an important role in the pathogenesis of tumor cell proliferation (24). Activation of EGFR stimulates its intrinsic tyrosine kinase activity and recruitment of cytosolic signaling proteins (28). The signals generated in the cytosol converge in the nucleus and activate cell cycle progression of breast tumor cells. EGF is a modest mitogen for MDA MB 231 breast cancer cells and we show slight induction of cell proliferation and G1 to S phase progression in these cells (Figs. 2 and 3). BMP-2 inhibited MDA MB 231 cell proliferation regardless of the presence of EGF (Figs. 1 and 2). Our results indicate that BMP-2 treatment arrests MDA cells at the G1 phase of cell cycle.

Nuclear targets of growth factor-mediated induction of cell proliferation are the cell cycle regulatory proteins (3–7). Cyclin D1 and E regulate the progression of cells in the G1 to S phase of cell cycle (5, 25). CDKs are regulated by cyclin kinase inhibitors (7, 8, 29). One of these proteins, p21, is a potent inhibitor of CDKs associated with cyclins D and E. It has been shown previously that p21 stimulates withdrawal from the cell cycle coupled to terminal differentiation (30). In breast carcinomas, increased expression of p21 was associated with relapse free survival (31). In addition to inhibition of CDKs, p21 inhibits DNA replication directly by binding to PCNA (32). In the present study we show that BMP-2 increases the level of p21 in MDA MB 231 breast tumor cells (Fig. 4). These data indicate that BMP-2-induced reduction in cell proliferation and S-phase entry may be due to the increased expression of p21 protein.

Increased cyclin D1-associated kinase activity is associated with increased proliferation of breast cancer cells (33). One of the mechanisms by which p21 blocks cell cycle progression is via interaction with cyclin D1 and cyclin E, subsequently resulting in inhibition of CDK activity (7–9). Our results show that BMP-2 increases the association of p21 with cyclin D1 and cyclin E in MDA MB 231 cells (Fig. 5) resulting in inhibition of their associated kinase activities (Fig. 6). Thus one of the mechanisms by which BMP-2 may inhibit MDA MB 231 cell proliferation is by inhibiting cyclin dependent kinases that are known to be activated in mid to late G1 and S phases of cell cycle.

During G1 phase of cell cycle the transcription factor E2F is associated with hypophosphorylated pRb. Cyclin dependent kinases phosphorylate pRb (34). pRb is also hyperphosphorylated in various breast cancer cells and tissues (35). Thus phosphorylated and inactivated pRb releases E2F transcrip-

tion factor which then activates transcription of a number of important genes necessary for cells to enter into S phase (34). In the present study we have shown that pRb is hyperphosphorylated in the presence of EGF in MDA MB 231 cells and BMP-2 causes reduction in the level of pRb phosphorylation in the presence and absence of EGF (Fig. 7). This observation provides one of the first mechanisms by which BMP-2 may inhibit MDA MB 231 breast cancer cell proliferation. Our findings may have important therapeutic implications in breast cancer treatment once the mechanism of action of BMP-2 has been characterized in more detail.

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Bone morphogenetic protein-2 induces cyclin kinase inhibitor p21 and hypophosphorylation of retinoblastoma protein in estradiol-treated MCF-7 human breast cancer cells

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Bone morphogenetic protein-2 induces cyclin kinase inhibitor p21 and hypophosphorylation of retinoblastoma protein in estradiol-treated MCF-7 human breast cancer cells

Nandini Ghosh-Choudhury [a,1,*], Goutam Ghosh-Choudhury [b,c,1], Anthony Celeste [d], Paramita M. Ghosh [a], Marissa Moyer [a], Sherry L. Abboud [b,c], Jeffrey Kreisberg [a,c]

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Abstract

The biologic effects and mechanisms by which bone morphogenetic proteins (BMPs) function in breast cancer cells are not well defined. A member of this family of growth and differentiation factors, BMP-2, inhibited both basal and estradiol-induced growth of MCF-7 breast tumor cells in culture. Flow cytometric analysis showed that in the presence of BMP-2, 62% and 45% of estradiol-stimulated MCF-7 cells progressed to S-phase at 24 h and 48 h, respectively. Estradiol mediates growth of human breast cancer cells by stimulating cyclins and cyclin-dependent kinases (CDKs). BMP-2 significantly increased the level of the cyclin kinase inhibitor, p21, which in turn associated with and inactivated cyclin D1. BMP-2 inhibited estradiol-induced cyclin D1-associated kinase activity. Also estradiol-induced CDK2 activity was inhibited by BMP-2. This inhibition of CDK activity resulted in hypophosphorylation of retinoblastoma protein thus keeping it in its active form. These data provide the first evidence by which BMP-2 inhibits estradiol-induced proliferation of human breast cancer cells.

Keywords: BMP-2; pRb; p21; Breast cancer cell

1. Introduction

17-β-Estradiol (estradiol) acts as a potent mitogen for breast epithelial cells and thus causes increased cell growth, both in vivo and in vitro. In estradiol-responsive human breast cancer cells like MCF-7, the hormone responsiveness is mediated by estrogen receptors (ERs) [1]. Activation of ER stimulates cyclin-dependent kinases (CDKs) to induce proliferation of MCF-7 cells. On the other hand, increased expression of cyclin kinase inhibitor p21 blocks CDK activity necessary for retinoblastoma protein (pRb) phosphorylation [2]. These results indicate that a concerted effect of different cell cycle proteins regulates cell cycle progression.

Bone morphogenetic proteins (BMPs), BMP 1–9, constitute a group of growth factors that are in-

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volved in ectopic bone formation [3]. They are produced as pro-mature forms, which are processed to active dimers of the mature region in a manner similar to the transforming growth factor β (TGFβ) [4]. Due to this similarity, BMPs are categorized as members of the TGFβ super family. In addition to other functions during embryonic development and limb formation, BMPs regulate chondrogenesis and osteogenesis [5–7]. Though BMPs have been detected in osteosarcomas and soft tissue carcinomas, the role of BMPs in breast cancer is still unclear. Like TGFβ receptors, multiple BMP receptors have recently been identified. They form two closely related groups known as type I and type II receptors, which contain multiple members. Both the receptor types have serine/threonine kinase activity in their cytoplasmic domains [8,9]. Three downstream target molecules for BMP-2 have recently been identified. These targets are Smad 1, Smad 5 and Smad 8. BMP-2 stimulates association of Smad 1 with the BMP receptor followed by phosphorylation of the Smad 1 C-terminus by the type I receptor [10,11]. Receptor-phosphorylated Smad 1 undergoes heterodimerization with the tumor suppressor protein Smad 4. This heterodimer then translocates to the nucleus and participates in transcription of genes [10,12].

Recently, Nakaoka et al. demonstrated that BMP-2 inhibits smooth muscle cell proliferation [13]. BMP-2 blocks serum and androgen-induced growth of human prostate cancer cells in culture [14]. We have recently shown that BMP-2 at a moderate dose blocked PDGF and EGF-induced DNA synthesis in primary glomerular mesangial cells without any effect on matrix gene expression [15,16]. Also a high dose of recombinant BMP-2 has recently been shown to inhibit soft agar growth of a variety of tumor samples including breast tumor [17]. However, the mechanism of BMP-2-mediated inhibition of tumor cell growth is not known. In this report, we demonstrate the inhibitory effect of BMP-2 on estradiol-induced MCF-7 human breast cancer cell proliferation in culture. BMP-2 increases the levels of cyclin kinase inhibitor p21 without any effect on estradiol-induced cyclin D1 expression. We also show that BMP-2 inhibits estradiol-induced cyclin D1-associated kinase and CDK2 activity with concomitant reduction of pRb phosphorylation. This is the first elucidation of the signaling mechanisms, involved in BMP-2-mediated inhibition of estradiol-induced breast cancer cell growth.

2. Materials and methods

Tissue culture materials were purchased from Gibco. Estradiol, phenyl methyl sulphonyl fluoride (PMSF), soybean inhibitor, leupeptin, myelin basic protein, propidium iodide and RNase A were obtained from Sigma. Histone H1 was purchased from Boehringer Mannheim. GST-pRb was obtained from Santa Cruz. Micro BCA reagent and enhanced chemiluminescence (ECL) kit were purchased from Pierce. Protein A-Sepharose CL 4B was purchased from Pharmacia. All antibodies were obtained from Santa Cruz. Recombinant BMP-2 was obtained from Genetics Institute.

MCF-7 breast cancer cells were obtained from Dr. Robert Klebe (Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio) and were routinely maintained in DMEM–F12 medium containing 10% new born calf serum. These MCF-7 cells are highly responsive to estradiol and tamoxifen. For experiments designed to test the mitogenic effect of estradiol, cells were grown in complete medium for 48 h to reach subconfluency and then placed in phenol red-free and serum-free DMEM for 48 h before addition of estradiol. Treatment with serum-free medium slows the growth of cells because they tend to arrest at G0/G1 phase. For cell cycle analysis near confluent cells were used for serum-deprivation to arrest in G0/G1 phase before addition of estradiol to release them.

2.1. Flow cytometric analysis

MCF-7 cells were trypsinized and washed with phosphate-buffered saline (PBS). The cells were fixed in 70% ethanol for 30 min at −20°C, centrifuged at 1500×*g* for 4 min, washed with PBS containing 1% bovine serum albumin (BSA) and resuspended in 150 μl PBS. For nuclear staining with propidium iodide, the cells were treated with 50 μl of 1 mg/ml RNase A (Sigma) followed by 100 μl of 100 μg/ml propidium iodide. The cells were incubated at 4°C for 18–24 h before they were analyzed by flow cytometry on FACStar Plus (Becton Dickinson Immunocytometry

Systems, San Jose, CA). Cells were illuminated with 200 mW of light at 488 nm produced by an argon-ion laser and the fluorescence was read using a 630/22 nm band-pass filter. Data were analyzed for 20000 viable cells as determined by forward and right angle light scatter and were stored as frequency histograms and subsequently analyzed by MODFIT software (Verity, Topsham, ME).

2.2. MTT (3-(4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide) assay for cell proliferation

Proliferation of MCF-7 cells in response to estradiol was determined using the MTT assay as described elsewhere [18]. In brief, 50 μl of 5 mg/ml MTT was added to the culture medium of growing cells (1 ml medium/well) and incubated for 4 h at 37°C in a humidified atmosphere with 5% CO_2. The medium was removed and 200 μl of DMSO was added to each well. The absorbance of the dissolved dye was measured at 540 nm.

2.3. Immunoprecipitation and immunoblotting

Immunoprecipitation was carried out according to methods described elsewhere, with minor modifications [16,19]. In brief, cells were lysed in immunoprecipitation buffer (50 mM Tris–HCl pH 7.5, 5 mM EDTA, 10 mM EGTA, 50 mM NaF, 20 mM β-glycerophosphate, 50 mM NaCl, 0.1% Nonidet P-40, 50 μg/ml PMSF, 10 μg/ml soybean trypsin inhibitor, 2 μg/ml leupeptin, 1 μg/ml aprotinin) and cleared of cell debris by centrifugation at 4°C. Protein estimation was done in supernatant by a micro BCA assay. 200 μg protein was routinely precleared by incubating with 20 μl of swelled protein A-Sepharose beads for 1 h in the cold. The cleared supernatant was immunoprecipitated at 4°C for 18–24 h using 1 μg of antibody followed by addition of 20 μl swelled protein A-Sepharose beads for 1 h. The protein A-Sepharose beads containing the antigen–antibody complex were then gently washed three times with immunoprecipitation buffer before eluting the bound proteins in the sodium dodecyl sulfate (SDS)–polyacrylamide gel loading buffer.

Immunoblotting was performed essentially as previously described [20]. Briefly, the cleared cell lysates or immunoprecipitates were separated in SDS–polyacrylamide gels (12% or 7.5% depending on the protein sizes). The proteins were electrophoretically transferred onto Nytran membrane. Following the transfer, the proteins were incubated with blocking solution (50 mM Tris–HCl pH 7.4–150 mM NaCl–0.2% Tween 20 (TBST) containing 5% non-fat dry milk) for 1 h at room temperature, followed by overnight incubation in primary antibody solution prepared in TBST containing 1% BSA. The membrane was subsequently washed five times in TBST for 5 min each, before the horseradish peroxidase-conjugated secondary antibody was added in TBST for 1 h at room temperature. The membrane was finally washed in TBST, five times for 5 min each and the antigen–antibody complex was detected using an ECL kit (Pierce) as per manufacturer's recommendations.

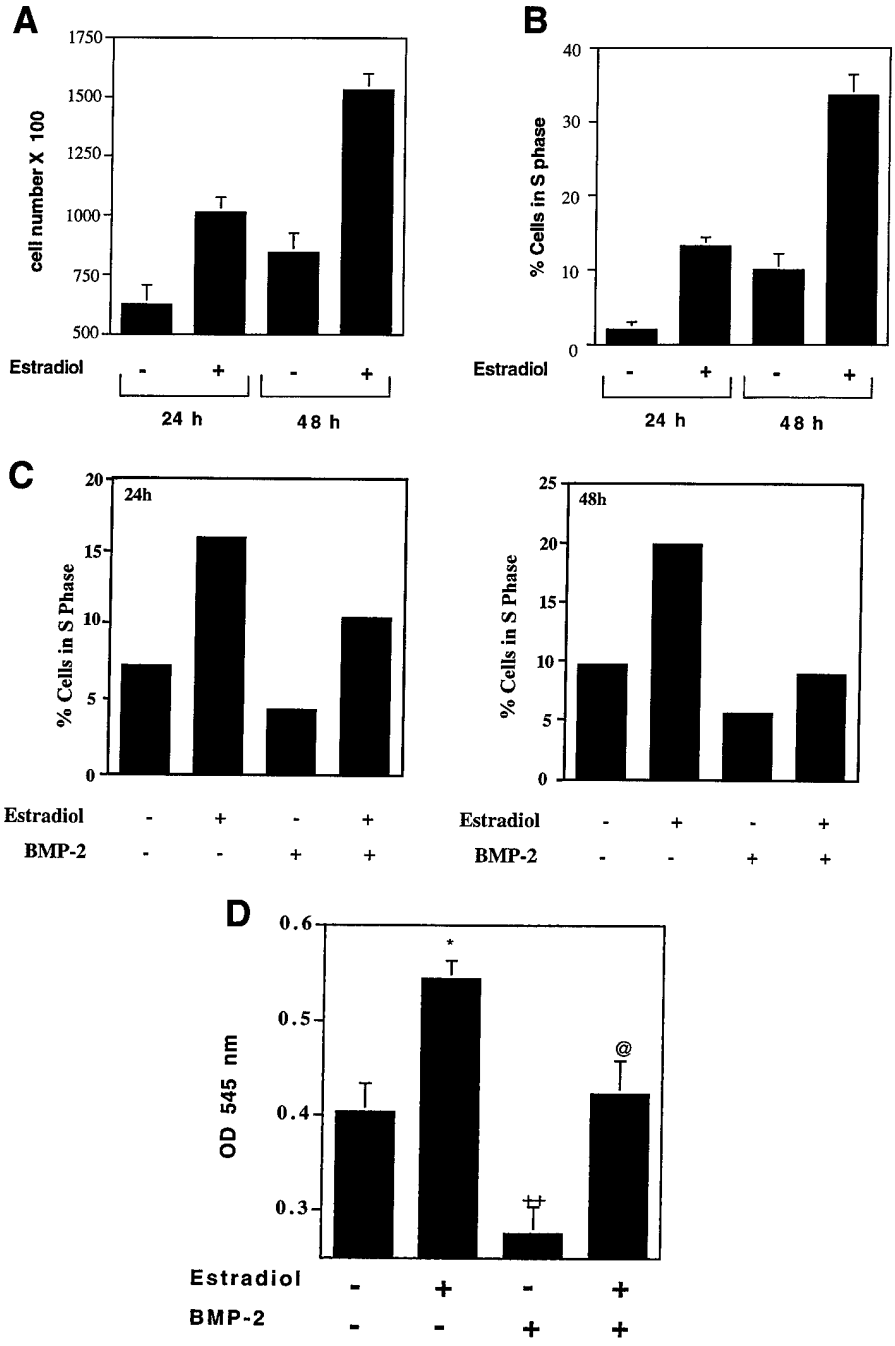
2.4. Cyclin D1-associated kinase and CDK2 assay

The assay was performed using the method of Gong et al. [21]. Briefly, cleared cell lysate was immunoprecipitated using antibody against cyclin D1 or CDK2 as described above. The immunecomplex beads were resuspended in kinase buffer (20 mM Tris–HCl pH 7.5 and 4 mM $MgCl_2$). To measure the cyclin D1-associated kinase activity, a fragment of pRb, that contains the in vivo phosphorylation sites, was used. For CDK2 activity, calf thymus histone H1 was used as a substrate. The reaction was carried out in the presence of 25 μM 'cold' ATP and 10 μCi [γ-^{32}P]ATP for 30 min at 37°C. The phosphorylated proteins were analyzed by SDS–polyacrylamide gel electrophoresis followed by autoradiography. Phosphorylation was quantitated using a densitometric scan of the phosphorylated bands in autoradiogram.

3. Results

3.1. Growth inhibition of MCF-7 cells by BMP-2

Estradiol is a potent mitogen for ER positive human MCF-7 breast carcinoma cells [22]. To establish the optimal conditions to assess the effect of BMP-2 on MCF-7 cell proliferation, MCF-7 cells were treated with 1 nM 17-β-estradiol for 24 and 48 h,



respectively, and the cell number was counted. As expected, estradiol increased the cell number at each time point (Fig. 1A). To examine the effect of estradiol on cell cycle progression, MCF-7 cells were subjected to flow cytometry. At 24 and 48 h, a significantly higher percentage of cells were in S-phase in the presence of estradiol, as compared to unstimulated control cells (Fig. 1B). At 48 h, 10% of cells entered the S-phase even in the absence of estradiol. This may be due to incomplete quiescence of MCF-7 breast tumor cells. An alternative possibility may be accumulation of mitogens in the culture medium during 48 h of incubation in the serum-deprived medium.

To determine the effect of BMP-2 on cell cycle progression of MCF-7 cells stimulated by estradiol,

Fig. 1. Effect of estradiol and BMP-2 on MCF-7 cell proliferation. (A) Confluent layers of MCF-7 cells in 35 mm tissue culture dishes were serum-starved for 48 h and incubated with 1 nM estradiol for 24 and 48 h. Cells in each dish were counted. Increase in cell number was 1.6- and 1.8-fold, respectively, at 24 and 48 h. (B) Flow cytometric analysis of estradiol-induced MCF-7 cell proliferation. Serum-deprived MCF-7 cells were grown in the presence or absence of 1 nM estradiol for 24 and 48 h. Cells were then trypsinized and analyzed by flow cytometry as described in Section 2. The percentage of cells in S-phase was plotted. The percentage of cells in S-phase was increased by 6.5- and 3.7-fold at 24 and 48 h, respectively. Means of triplicate determinations are shown in A and B. (C) Quantitation of flow cytometric analysis of cells treated with BMP-2 and estradiol. MCF-7 cells were treated with 100 ng/ml BMP-2 and 1 nM estradiol for 24 h (left panel) and 48 h (right panel) before subjecting them to flow cytometric analysis. The percentage of cells in S-phase was plotted for each condition. (D) Effect of BMP-2 on estradiol-induced MCF-7 cell proliferation. 48 h serum-deprived MCF-7 cells were treated with 100 ng/ml BMP-2 and 1 nM estradiol. MTT assay was performed as an index of cell proliferation as described in Section 2. Results are means ± S.E.M. of three independent experiments. $*P < 0.05$ vs. untreated cells. $++P < 0.05$ vs. untreated control. $@P < 0.05$ vs. estradiol-treated cells.
←

cells were incubated with estradiol for 24 and 48 h, either in presence or absence of BMP-2. BMP-2 inhibited estradiol-induced S-phase progression of these cells. Quantitation of these results shows that only 62% and 45% of estradiol-treated MCF-7 cells entered S-phase at 24 and 48 h, respectively, in the presence of BMP-2 (Fig. 1C, left and right panels). BMP-2 alone also inhibited S-phase entry of control cells, by 41% and 43% at 24 and 48 h, respectively. To determine if the effect of BMP-2 on estradiol-induced cell cycle progression correlated with cell growth, MTT assays were performed. Fig. 1D shows that BMP-2 significantly inhibited estradiol-stimulated as well as basal MCF-7 cell proliferation. A photomicrograph of MCF-7 cells in the absence and presence of BMP-2 is shown in Fig. 2. As evident, treatment of these cells for 48 h with BMP-2 does not have any toxic effect. Taken together, these results indicate that BMP-2 inhibits estradiol-induced cell growth by preventing the entry of MCF-7 cells into S-phase.

3.2. BMP-2 stimulates expression of cyclin kinase inhibitor, p21, in estradiol-treated MCF-7 cells

Progression of the cell cycle is regulated by a series of CDKs [23]. These serine/threonine kinases are positively regulated by cyclins [24,25]. One of the G1 phase cyclins, cyclin D1, is overexpressed in more than 50% of human breast adenocarcinomas [26–28]. We studied the effect of estradiol on cyclin D1 expression. In accordance with the previous report [29], estradiol treatment of MCF-7 cells increased the level of cyclin D1 (Fig. 3A, compare lane 2 with lane 1). However, pretreatment of MCF-7 cells with BMP-2 had no significant effect on estradiol-induced expression of cyclin D1 (Fig. 3A, compare lane 4 with lane 2). These data indicate that the effect of BMP-2 on estradiol-induced MCF-7 cell proliferation is not caused by the modulation of cyclin D1 levels during cell cycle progression.

CDK activity is also regulated by cyclin kinase inhibitors [25]. One such protein, p21, is a universal

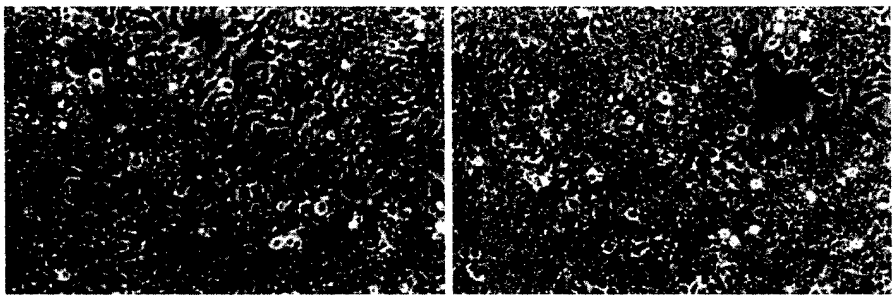


Fig. 2. Photomicrograph of MCF-7 cells in the presence and absence of BMP-2. Serum-deprived MCF-7 cells were incubated with BMP-2 for 48 h before taking the photograph. The phase contrast photomicrograph is shown.

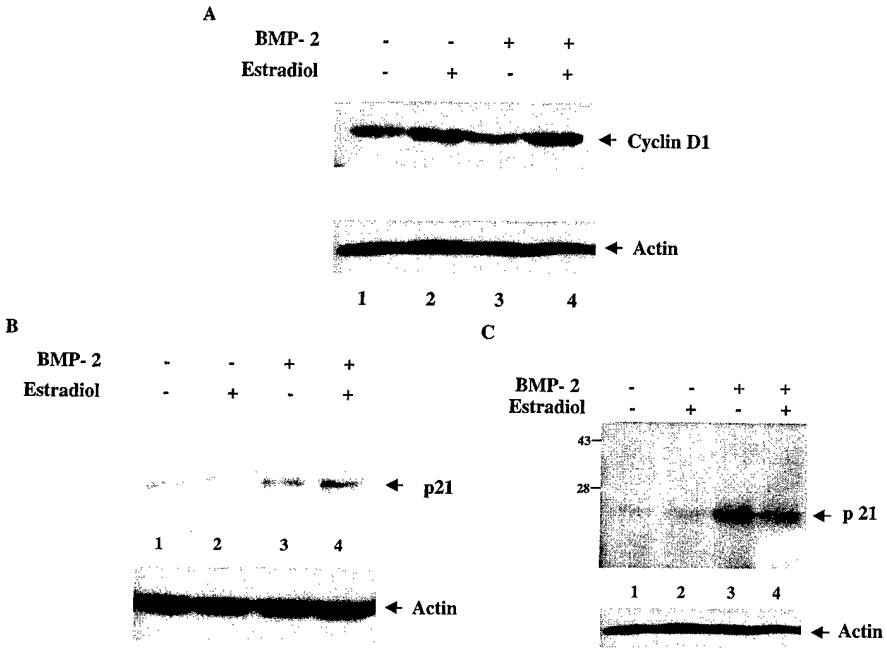


Fig. 3. (A) Effect of BMP-2 on estradiol-induced expression of cyclin D1 and p21. MCF-7 cells serum-deprived for 48 h were incubated with 1 nM estradiol in the presence and absence of 100 ng/ml BMP-2 for subsequent 48 h. Cleared cell lysates were analyzed by immunoblotting with cyclin D1. (B and C) Effect of BMP-2 on p21 expression. Serum-deprived MCF-7 cells were incubated with estradiol in the presence and absence of 100 ng/ml BMP-2 for 6 h (B) and 48 h (C), respectively. The lysates were immunoblotted with p21 antibody and the signal was developed by ECL. Lower panels show immunoblotting of the same lysates with anti-actin antibody to demonstrate equal loading of proteins in each lane.

inhibitor of CDKs, that interacts with multiple cyclin–CDK complexes. It thereby inhibits their kinase activity, which drives the cells through the cell cycle [2,30,31]. To understand the mechanism of BMP-2 inhibition of cell cycle progression, we studied its effect on p21 expression. MCF-7 cells were incubated with BMP-2 and estradiol for 6 and 48 h. At both time points, estradiol did not have any affect on p21 protein expression as determined by immunoblot analysis (Fig. 3B,C, compare lanes 2 with lanes 1 in both panels). In contrast, treatment of MCF-7 cells with BMP-2 alone significantly increased the level of p21 protein expression (Fig. 3B,C, compare lanes 3 with lanes 1). In the cells co-treated with BMP-2 and estradiol (lane 4), the level of p21 expression remained increased as compared to untreated and estradiol-treated cells. These data indicate that the inhibitory effect of BMP-2 on MCF-7 cell proliferation may partly be due to its effect on increased expression of p21.

3.3. *BMP-2 inhibits cyclin D1-associated kinase activity via p21*

For p21 to exert its inhibitory effect on cell cycle progression, it must associate with one of the cyclin–CDK complexes [24,25]. Since estradiol-induced increase in cyclin D1 levels resulted in cell progression, while BMP-2-induced increase in p21 levels caused G1 arrest, we analyzed the association of p21 with cyclin D1 under similar conditions. Lysates of serum-deprived MCF-7 cells treated with estradiol in the presence and absence of BMP-2 were immunoprecipitated with the antibody to p21, followed by immunoblotting with a cyclin D1 antibody. As shown in Fig. 4A, estradiol alone has no effect on association of cyclin D1 with p21. In contrast, p21 was found to be associated with cyclin D1 in cells treated with BMP-2 alone or in combination with estradiol (Fig. 4A, lanes 3 and 4). These data indicate that BMP-2 treatment causes an increased association of p21 with

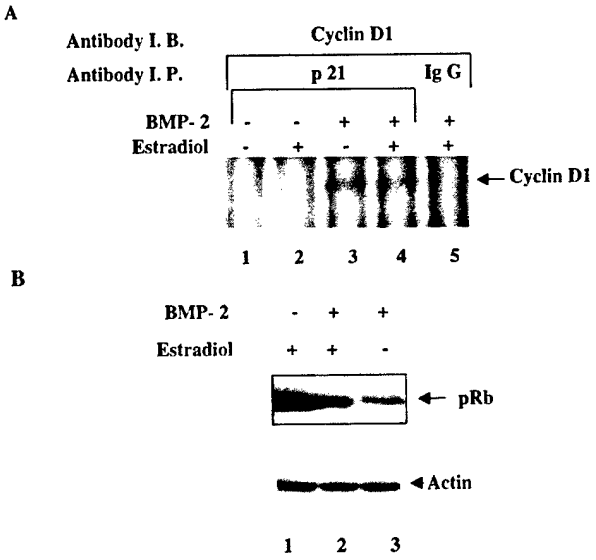


Fig. 4. (A) Effect of BMP-2 on association of p21 with cyclin D1. Cleared cell lysates from MCF-7 cells, treated as described in Fig. 3B,C, were immunoprecipitated (I.P.) with either anti-p21 or control IgG. The immunoprecipitated proteins were eluted from the immunebeads, separated on a 12% SDS–polyacrylamide gel and were immunoblotted (I.B.) with anti-cyclin D1 antibody. Lanes 1–4 represent p21 immunoprecipitates. Lane 5 shows IgG immunoprecipitates. (B) Effect of BMP-2 on estradiol-induced cyclin D1-associated kinase activity. Serum-deprived MCF-7 cells were incubated with estradiol in the presence of BMP-2. The lysates were immunoprecipitated with cyclin D1 antibody. The immunoprecipitates were assayed for kinase activity in the presence of [γ-^{32}P]ATP using pRb as substrate. The labeled protein was separated by SDS gel electrophoresis and visualized by autoradiography. The lower panel shows an immunoblot of same samples with actin antibody. →

cyclin D1 in MCF-7 cells which may result in the inhibition of cyclin D1-dependent kinase activity.

More recently, a role for p21 has been described as the assembly factor for cyclin D and CDK4/6 [32,33].

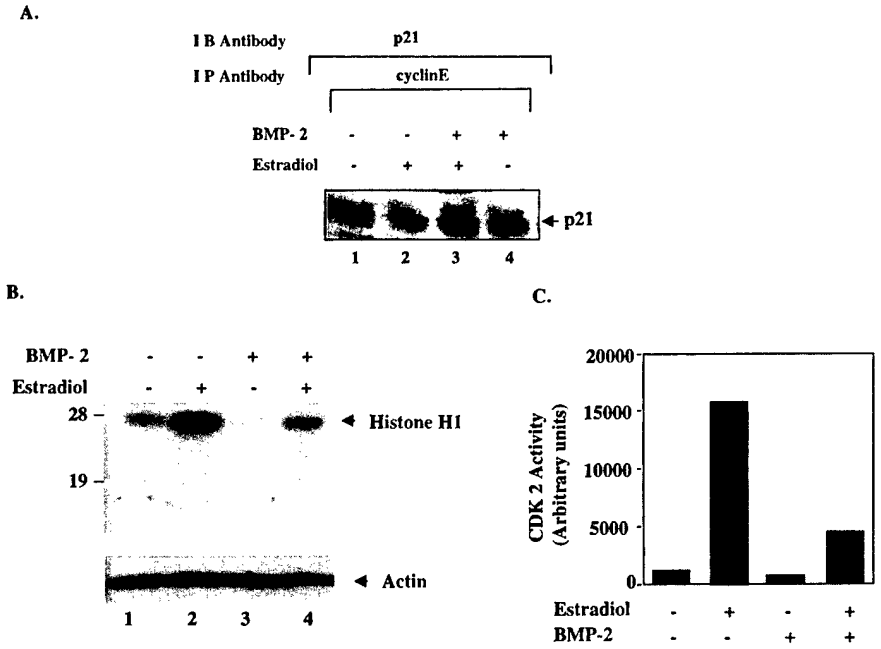


Fig. 5. (A) Effect of BMP-2 on association of p21 with cyclin E. Cleared cell lysates from MCF-7 cells, treated as described in Fig. 4, were immunoprecipitated (I.P.) with anti-cyclin E. The immunoprecipitated proteins were eluted from the immunebeads, separated on a 15% SDS–polyacrylamide gel and were immunoblotted (I.B.) with anti-p21 antibody. Effect of BMP-2 on estradiol-induced CDK2 activity. (B) The cleared cell lysates from MCF-7 cells treated as described in Fig. 4 were immunoprecipitated with an anti-CDK2 antibody. The washed immunebeads were used in an in vitro immunecomplex kinase assay with histone H1 as substrate in the presence of [γ-^{32}P]ATP. The labeled proteins were separated by SDS gel electrophoresis and visualized by autoradiography. The lower panel shows immunoblotting of the same lysates with anti-actin antibody. (C) Quantitation of histone H1 phosphorylation. The radioactivity incorporated into histone H1 in (A) was measured by a densitometric scan as described in Section 2 and plotted as histogram.

Another role of p21 in cell cycle is its inhibitory effect on CDK activity. Increased expression of p21 has been shown to inhibit both cyclin D1 and cyclin E-associated kinases [2]. To test this, lysates of MCF-7 cells treated with BMP-2 and estradiol were immunoprecipitated with cyclin D1 antibody. The immunoprecipitates were assayed for D1-associated kinase activity using pRb as in vitro substrate. As shown in Fig. 4B, BMP-2 inhibited estradiol-induced cyclin D1-associated kinase activity (compare lane 2 with lane 1). These data indicate that the inhibitory effect of BMP-2 may involve reduced pRb phosphorylation by cyclin D1-associated kinase (see below).

3.4. BMP-2 inhibits estradiol-induced CDK2 kinase activity and pRb phosphorylation

In the late G1 phase of cell cycle progression, E-type cyclin regulates CDK activity which is necessary for cells to enter and proceed through the S-phase [34]. p21 has been shown to regulate cyclin E via physical association. Since the p21 level was increased by BMP-2 (Fig. 3B,C), we tested if recombinant BMP-2 regulates p21 association with cyclin E. Cyclin E immunoprecipitates from lysates of estradiol or BMP-2 plus estradiol-treated MCF-7 cells were immunoblotted with p21 antibody. The results show that BMP-2 stimulated increased association of p21 with cyclin E in the presence and absence of estradiol as compared to estradiol alone (Fig. 5A, compare lanes 3 and 4 with lane 2). During late G1 and S-phase, cyclin E regulates CDK2 activity. To understand the mechanism of regulation of CDK2 in MCF-7 breast cancer cells, we analyzed the kinase activity associated with CDK2 in cells treated with estradiol in the presence or absence of BMP-2. Cell lysates were immunoprecipitated with a CDK2 antibody. The immunebeads were then used in an in vitro immunecomplex kinase assay with histone H1 as substrate in the presence of [γ-^{32}P]ATP. The data showed increased phosphorylation of histone H1 by CDK2 in cells treated with estradiol (Fig. 5B, compare lane 2 with 1). Estradiol-induced CDK2 activity was significantly inhibited by BMP-2 (Fig. 5B, compare lane 4 with lane 2). Quantitation of histone H1 phosphorylation showed 11-fold increase in CDK2 activity in the presence of estradiol (Fig. 5C), and BMP-2 inhibited 70% of estradiol-induced CDK2 activity (Fig. 5C). BMP-2 also partially inhibited the basal activity of CDK2 in MCF-7 cells. These data indicate that BMP-2-mediated inhibition of estradiol-induced MCF-7 proliferation may in part be due to its inhibitory effect on CDK2 activity.

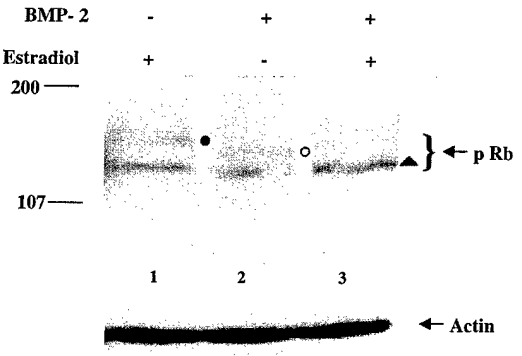


Fig. 6. Effect of BMP-2 on estradiol-induced pRb phosphorylation. The cleared cell lysates of MCF-7 cells, treated with estradiol in the presence and absence of BMP-2, were immunoblotted with an anti-pRb antibody. The migration of molecular weight markers (in kDa) is shown in the left margin. The filled circle shows hyperphosphorylated pRb in the highest phosphorylated form. The open circle shows pRb in intermediate phosphorylated form and the filled triangle shows pRb in hypophosphorylated form.

One of the targets of CDKs during cell cycle progression is pRb [24,35]. Hypophosphorylated pRb is active and inhibits cell cycle progression. Proliferative signals integrate into the nucleus to induce CDK-dependent phosphorylation of pRb rendering pRb inactive and resulting in DNA synthesis [35]. We showed in Fig. 4B that in in vitro kinase assay, pRb phosphorylation is reduced by BMP-2. To study the effect of BMP-2 on estradiol-induced pRb phosphorylation in MCF-7 cells, we analyzed lysates of MCF-7 cells by phosphorylation-dependent mobility shift assay. The degree of pRb phosphorylation is determined by its electrophoretic mobility, with hyperphosphorylated pRb forms migrating slower than the hypophosphorylated form in SDS gel. Lysates from MCF-7 cells, treated with estradiol or BMP-2 alone or with estradiol in the presence of BMP-2, were immunoblotted with an anti-pRb antibody. As shown in Fig. 6, estradiol caused hyperphosphorylation of pRb as indicated by the slower migration of this protein (lane 1, indicated by filled circle). Treatment of MCF-7 cells with BMP-2 alone resulted in the partial phosphorylation of pRb (lane 2, indicated

by open circle). In contrast, BMP-2 significantly inhibited hyperphosphorylation of pRb induced by estradiol and only hypophosphorylated pRb was the predominant form detected (Fig. 6, lane 3, indicated by filled triangle). These data indicate that the observed growth inhibitory effect of BMP-2 in estradiol-induced MCF-7 breast cancer cell proliferation could be caused by decreased CDK-dependent pRb phosphorylation.

4. Discussion

Our study demonstrates an inhibitory effect of BMP-2 on estradiol-induced MCF-7 breast cancer cell proliferation. BMP-2 stimulates increased expression of p21 cyclin kinase inhibitor. Consistent with this idea is our observation showing inhibition of estradiol-induced cyclin D1-associated kinase and CDK2 activity in response to BMP-2. Finally, we provide the first evidence that BMP-2 maintains the pRb tumor suppressor protein in a partially phosphorylated form.

Binding of estrogen to its receptor regulates a cohort of responsive genes that appears to regulate cell cycle progression. CDK4 and CDK6 form complexes with D-type cyclins during mid and late phases of G1, while CDK2 binds to cyclin E and D during late G1 [36–38]. One link between proliferative signals and cell cycle progression is provided by the induction of the secondary response genes, such as cyclin D1, following mitogenic stimulation [36]. In breast cancer, chromosome 11q13, which contains the cyclin D1 gene, has been shown to be amplified preferentially in ER positive tumors [39,40]. It has also been suggested that overexpression of cyclin D1 in MCF-7 cells causes them to proliferate in growth factor-deprived conditions [41]. In simvastatin or lovastatin-arrested MCF-7 human breast cancer cells, estrogen stimulates cell cycle entry by increasing cyclin D1 expression [42]. This effect of estrogen was due to transcriptional activation of the cyclin D1 gene by an estrogen-regulated response region present between the −944 bp of upstream sequences and the transcription start site of the cyclin D1 gene [42]. Cyclins, in association with CDKs and cyclin kinase inhibitors, control cell cycle progression through different phases of transitions and checkpoints. One of the cyclin kinase inhibitors, p21, has been shown to stimulate withdrawal from the cell cycle coupled to terminal differentiation [43]. Immunohistochemical analysis of breast carcinomas has shown that increased expression of p21 was associated with relapse-free survival [44]. p21 inhibits all the CDKs associated with cyclins A, D1 and E that are required for G1/S progression [31,45]. In addition to CDK inhibition, and thereby blocking cells from entering S-phase, p21 inhibits the DNA replication directly by binding to PCNA [46]. In the present study, we show that estradiol-induced S-phase entry of MCF-7 breast carcinoma cells is inhibited by the growth and differentiation factor BMP-2 (Fig. 1). Furthermore, our results demonstrate that BMP-2 causes increase in the levels of p21 protein as early as 6 h which sustains until 48 h (Fig. 3B,C). These data indicate that our observation of BMP-2-induced reduction in S-phase entry (Fig. 1C) and reduced proliferation (Fig. 1D) may be due to the increased expression of p21 protein (Fig. 3B,C). One of the mechanisms by which p21 blocks cells from entering S-phase is via interaction with cyclin D1 during G1 phase of the cell cycle, subsequently resulting in inhibition of CDK4 activity [2]. In the present study, we demonstrate association of p21 with cyclin D1 in the presence of BMP-2 (Fig. 4A). This may be the cause of reduced cyclin D1-associated kinase activity (Fig. 4B).

In addition to activation of cyclin D1/CDK4 during G1 phase, activation of cyclin E/CDK2 in late G1 is required for cells to progress through the cell cycle [38,47]. p21 inhibits both cyclin D1/CDK4 activity and cyclin E/CDK2 activity [2,30,48]. Treatment of MCF-7 breast cancer cells with estradiol stimulates cyclin D1-associated kinase (Fig. 4B) and CDK2 activity (Fig. 5B), which confirms the previous finding [47]. Pretreatment of cells with BMP-2, however, significantly blocked the estrogen-induced increase in both these kinase activities (Figs. 4B and 5B). Furthermore, BMP-2 increased the association of p21 with cyclin E (Fig. 5A). Our data for the first time demonstrate that BMP-2 targets the cell machinery at the level of CDKs. Thus one of the mechanisms by which BMP-2 inhibits MCF-7 cell proliferation is by inhibiting CDKs that are known to be activated in mid to late G1 and S-phases of cell cycle.

One of the targets of G1 CDKs is the tumor suppressor protein pRb [35]. This notion is established from various in vitro and in vivo studies. Cyclin D1/CDK4 complex can phosphorylate pRb in vitro [49]. The physiologic regulators that intercept CDK4/6 activity also block pRb phosphorylation. Similarly, overexpression of cyclin E in human osteosarcoma cells increases pRb phosphorylation [50]. pRb is also hyperphosphorylated in various breast cancer cells and tissues by cyclin E/CDK2 activity [51]. In tamoxifen-arrested MCF-7 cells, estradiol stimulates cyclin E/CDK2-dependent pRb phosphorylation [47]. We have also shown that treatment of serum-deprived MCF-7 cells with estradiol increased the level of hyperphosphorylated inactive pRb and that presence of BMP-2 during estradiol treatment caused reduction in the degree of pRb phosphorylation (Fig. 6). These observations describe one of the first mechanisms by which BMP-2 may inhibit MCF-7 breast cancer cell growth in culture.

In summary, we have demonstrated that BMP-2 inhibits estradiol-induced proliferation of human breast cancer cells. This effect of BMP-2 appears to be mediated by inhibition of positive cell cycle regulatory proteins. Hyperproliferation of estrogen-responsive breast cancer cells is one of the major causes of tumor formation in early stages of breast cancer. Agents such as BMP-2 that inhibit estradiol-induced breast cancer cell proliferation may prove to be important therapeutic tools once their mechanisms of action are more thoroughly characterized.

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Requirement of BMP-2-induced Phosphatidylinositol 3-Kinase and Akt Serine/Threonine Kinase in Osteoblast Differentiation and Smad-dependent *BMP-2* Gene Transcription*

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The mechanism by which bone morphogenetic protein-2 (BMP-2) induces osteoblast differentiation is not precisely known. We investigated the involvement of the phosphatidylinositol (PI) 3-kinase/Akt signal transduction pathway in modulation of this process. BMP-2 stimulated PI 3-kinase activity in osteogenic cells. Inhibition of PI 3-kinase activity with the specific inhibitor Ly-294002 prevented BMP-2-induced alkaline phosphatase, an early marker of osteoblast differentiation. Expression of dominant-negative PI 3-kinase also abolished osteoblastic induction of alkaline phosphatase in response to BMP-2, confirming the involvement of this lipid kinase in this process. BMP-2 stimulated Akt serine/threonine kinase activity in a PI 3-kinase-dependent manner in osteoblast precursor cells. Inhibition of Akt activity by a dominant-negative mutant of Akt blocked BMP-2-induced osteoblastic alkaline phosphatase activity. BMP-2 stimulates its own expression during osteoblast differentiation. Expression of dominant-negative PI 3-kinase or dominant-negative Akt inhibited BMP-2-induced BMP-2 transcription. Because all the known biological activities of BMP-2 are mediated by transcription via BMP-specific Smad proteins, we investigated the involvement of PI 3-kinase in Smad-dependent BMP-2 transcription. Smad5 stimulated BMP-2 transcription independent of addition of the ligand. Dominant-negative PI 3-kinase or dominant-negative Akt inhibited Smad5-dependent transcription of BMP-2. Furthermore dominant-negative Akt inhibited translocation of BMP-specific Smads into nucleus. Together these data provide the first evidence that activation of BMP receptor serine/threonine kinase stimulates the PI 3 kinase/Akt pathway and define a role for this signal transduction pathway in BMP-specific Smad function during osteoblast differentiation.

Bone morphogenetic proteins (BMPs),[1] a group of polypeptides within the transforming growth factor (TGF)-β superfamily, were originally identified by their ability to induce endochondral bone formation in ectopic extraskeletal sites *in vivo* (1). BMPs stimulate differentiation of pluripotent mesenchymal cells into the osteogenic lineage and enhance the differentiated function of osteoblasts. Among many other BMPs, BMP-2 induces differentiation of preosteoblasts into mature osteoblasts by regulating signals that stimulate a specific transcriptional program required for bone formation (2, 3).

Similar to TGF-β, BMPs exert their effect via type I and type II transmembrane serine/threonine kinase receptors (4, 5). The type II receptor binds the ligand with high affinity. The type I receptors, Alk3 (BMPRIA) and Alk6 (BMPRIB) are mainly responsible to transduce the signal, although they have weak BMP binding properties (6). Binding of BMP to its receptors induces phosphorylation of the type I receptor in its GS domain and recruitment of receptor-specific Smads (4, 5, 7). Upon phosphorylation, Smad1, or one of its close homologs Smad5 and Smad8, heterodimerizes with the common Smad, Smad4. This complex translocates to the nucleus where it associates with transcriptional coactivators and acts as a transcription factor to regulate tissue or cell type-specific genes required for the divergent biological functions of BMPs (4, 5, 7). In osteogenic cells, BMP-specific Smads stimulate expression of *Cbfa1*, the only known osteoblast-specific transcription factor to induce genes required for mature osteoblast differentiation and maintenance (8, 9).

Differentiation in response to extracellular factors depends on the action of kinases including both tyrosine and serine/threonine kinases (10, 11). In the case of neuronal differentiation, the tyrosine kinase activity of nerve growth factor (NGF) receptor and its downstream target ERK1/2-type of mitogen-activated serine/threonine protein kinase are essential (12). Recently a critical role for a lipid kinase, phosphatidylinositol 3-kinase (PI 3-kinase), has been suggested in muscle and adipocyte differentiation (13, 14). Activation of PI 3-kinase is often associated with increased tyrosine phosphorylation induced by growth and differentiation factors (15). PI 3-kinase functions as the focal point in cellular signaling leading to cell growth, regulating cytoskeletal structure, and preventing apoptosis (16–18). Involvement of PI 3-kinase has recently been shown to

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[1] The abbreviations used are: BMP, bone morphogenetic protein; BMPR, BMP receptor; PI, phosphatidylinositol; DN, dominant-negative; HA, hemagglutinin; TGF, transforming growth factor; GFP, green fluorescence protein; ERK, extracellular signal-regulated kinase; pNPP, *p*-nitrophenylphosphate; LUC, luciferase.

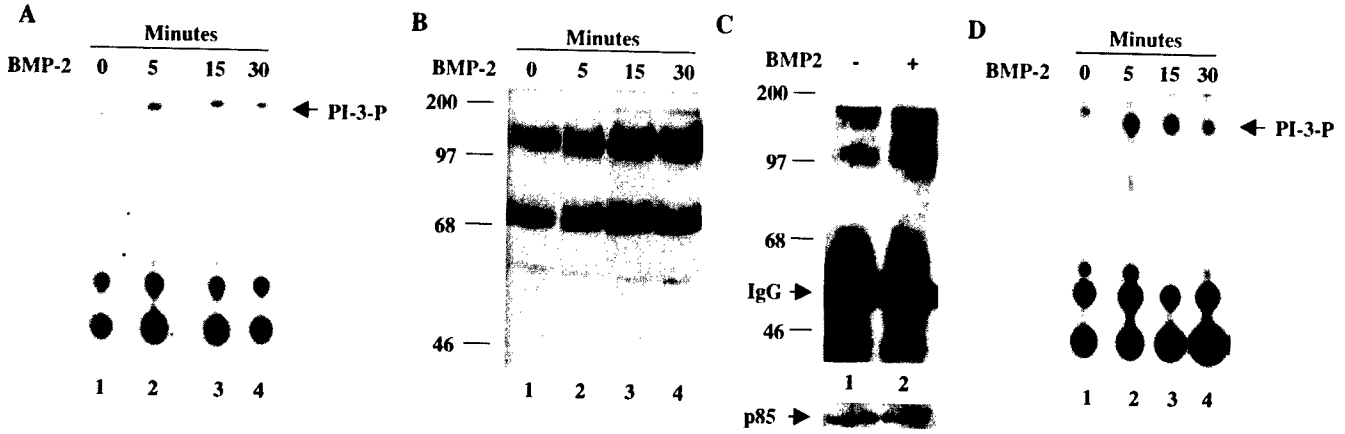

FIG. 1. **BMP-2 stimulates PI 3-kinase activity in 2T3 osteoblast precursor cells.** *A*, serum-deprived 2T3 cells were incubated with BMP-2 for indicated periods of time. Cleared cell lysates were immunoprecipitated with anti-p85 antibody followed by PI 3-kinase assay as described under "Experimental Procedures." The 3-phosphorylated PI (*arrow*) was separated by TLC. *B*, 50 μg of 2T3 cell lysates were analyzed by 10% SDS-polyacrylamide gel. Separated proteins were transferred to nitrocellulose membrane and immunoblotted with anti-phosphotyrosine antibody as described (25, 28–31). *C*, 500 μg of 2T3 cell lysate were immunoprecipitated with anti-p85 antibody followed by anti-phosphotyrosine immunoblotting. *D*, PI 3-kinase assay was performed with anti-phosphotyrosine immunoprecipitates from lysates of BMP-2-treated 2T3 cells and analyzed by TLC. *Arrow* indicates position of PI 3-phosphate (*PI-3-P*).

regulate some of the biological properties of TGF-β such as epithelial-mesenchymal transition and matrix protein expansion (19). However, the role of PI 3-kinase has not been investigated in osteogenesis, especially in response to BMPs. In this study, we show that BMP-2 stimulates tyrosine phosphorylation and PI 3-kinase activity in osteogenic cells. We demonstrate the requirement of PI-3 kinase and its downstream target, Akt serine/threonine kinase, for BMP-2-induced expression of an osteoblast differentiation marker alkaline phosphatase, and for BMP-2 transcription. Finally we demonstrate that a cross-talk exists between BMP-specific Smad and PI 3-kinase/Akt pathway to induce transcription of BMP-2, a necessary growth and differentiation factor for osteogenic cells.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture reagents and LipofectAMINE were obtained from Invitrogen. Recombinant BMP-2 was obtained from the Genetics Institute. Nonidet P-40, phenylmethylsulfonyl fluoride, and Na_3VO_4 were purchased from Sigma. Aprotinin was obtained from Bayer. Antibody against the p85 subunit of PI 3-kinase, BMPRI, and Smads were purchased from Santa Cruz Biotechnology. Monoclonal anti-phosphotyrosine antibody (4G10) and Akt antibody were obtained from UBI Inc. Anti-hemagglutinin (HA) antibody was purchased from Babco. Histone H2B was purchased from Roche Molecular Biochemicals. Anti-phospho-Akt antibody was from Cell Signaling. Dual luciferase assay kit was purchased from Promega Inc. The pSRαΔp85 plasmid encoding for a dominant-negative (DN) subunit for PI 3-kinase was a gift from Dr. Wataru Ogawa, Kobe University, Japan. The dominant-negative pCMV6-HA-Akt (K179M) expression vector was a kind gift from Dr. Thomas Franke, Harvard University. Smad1 plasmid was a gift from Dr. Joan Massague, Memorial Sloan-Kettering Cancer Center, NY. Adenovirus vectors containing constitutively active HA-tagged Alk3 Q233D (Ad-BMPRIA-QD) and Alk6 Q203D (Ad-BMPRIB-QD) were kindly provided by Anita Roberts, National Cancer Institute, MD.

Cell Culture and Adenovirus Infection—2T3 cells were isolated and cloned from a transgenic mouse with *BMP-2* promoter-driven SV-40 T antigen (20). These cells are responsive to BMP-2, undergo bone matrix formation *in vitro*, and have been extensively characterized recently (20–23). 2T3 cells are routinely grown in α-minimum Eagle's medium with 10% fetal bovine serum. The C2C12 multipotent cells were grown in Dulbecco's modified Eagle's medium in the presence of 10% fetal bovine serum. For osteoblastic differentiation, the cells were grown in the absence of serum, with or without recombinant BMP-2 as previously described (24). In experiments involving expression of constitutively active BMP receptor (BMPR), 2T3 cells in serum-free medium were infected with 100 m.o.i. Ad-BMPRIA-QD or Ad-BMPRIB-QD at room temperature for 1 h. Medium was changed with fresh serum-free medium. Similarly the 2T3 cells were infected with adenovirus vector expressing dominant-negative HA-tagged Akt (Ad-DN-Akt) essentially as described before (25). For all adenovirus infection, Ad-GFP containing green fluorescence protein was used as a control.

Immunoprecipitation, Immunoblotting, and PI 3-Kinase and Akt Kinase Assay—Cells were lysed in radioimmune precipitation assay buffer (RIPA) (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 0.05% aprotinin, and 1% Nonidet P-40) at 4 °C for 30 min. Cell extracts were centrifuged for 30 min at 4 °C. Protein concentration was determined in the supernatant using BioRad reagent. PI 3-kinase was assayed using PI as substrate in the presence of [γ-^{32}P]ATP and the PI 3-phosphate was separated by thin-layer chromatography as described (26, 27). RIPA lysates were used to immunoprecipitate Akt, and immunocomplex kinase assay was performed using histone H2B as substrate and [γ-^{32}P]ATP in kinase assay buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 25 mM β-glycerophosphate, 2 mM dithiothreitol, 1 mM Na_3VO_4, and 5 μM ATP). The phosphorylated histone H2B was separated by 15% SDS-polyacrylamide gel and visualized by autoradiography (25). Immunoblotting of the lysates was performed using appropriate antibodies as described previously (25, 28–31).

Alkaline Phosphatase Staining and Activity—The cells were fixed in 10% formalin and stained using 5-bromo-4-chloro-3-indoyl phosphate and nitro blue tetrazolium essentially as described previously (20, 32). The stained structures were examined and photomicrographed (×200) using Nikon digital camera attached to the microscope. Lysates from 2T3 cells was assayed for alkaline phosphatase activity using *p*-nitrophenylphosphate (pNPP) as substrate essentially as described (32).

Transfection and Luciferase Assay—The BMP-2-LUC is a reporter plasmid in which the firefly luciferase cDNA is expressed from a 2.7-kb 5′-flanking sequence of the *BMP-2* gene (3, 20, 33). This BMP-2-LUC reporter plasmid was transfected into cells along with vector or different dominant-negative expression plasmids using LipofectAMINE reagent as described (25, 28, 30, 31, 33). To correct for transfection efficiency a CMV-*Renilla* luciferase plasmid was included. Luciferase activity in the lysate was determined using a dual luciferase assay kit.

Immunofluorescence Staining—2T3 cells in 8-well chamber slides were infected with 100 m.o.i. Ad-DN-Akt for 24 h following incubation with BMP-2 for 30 min. The cells were fixed in cold methanol for 15 min, blocked with donkey IgG for 15 min, and then incubated with primary antibody, which recognizes BMP-specific Smad5 and Smad1 for 30 min. Stained cells were treated with fluorescein isothiocyanate-tagged secondary antibody for 30 min. The cells were then viewed by a Zeiss Axiophot microscope and photographed using SPOT software.

RESULTS

Activation of PI 3-Kinase in Response to BMP-2—To investigate the role of PI 3-kinase in BMP-2-induced osteoblast differentiation, we used the 2T3 cells, which we established from the calveria of a mouse expressing an SV-40 T-antigen transgene under transcriptional control of the *BMP-2* promoter (20). This group and others (20–23) have shown that these cells turn on an osteoblast-specific gene program in response to BMP-2 and undergo differentiation to form mature osteoblasts. 2T3

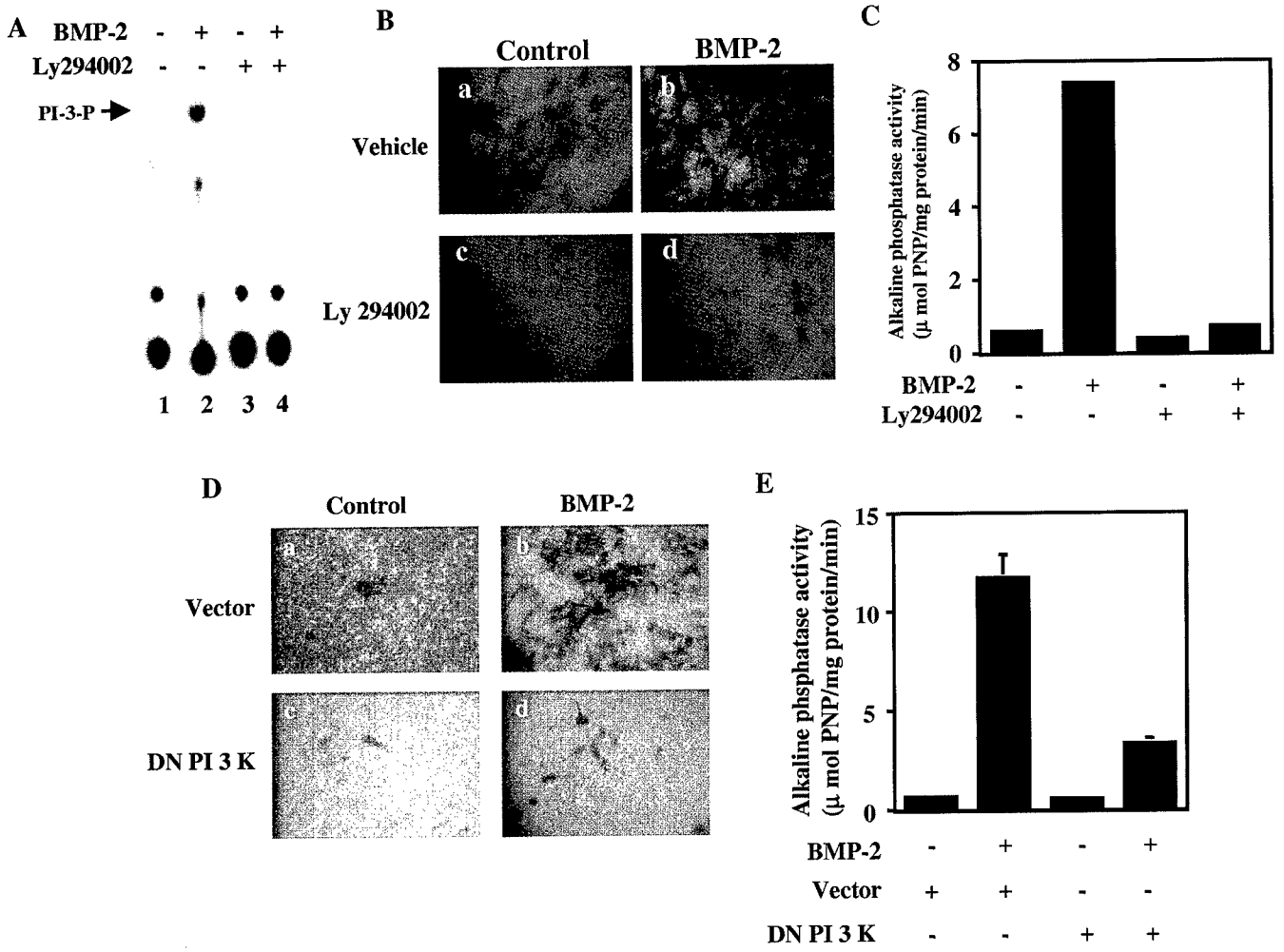

FIG. 2. **Inhibition of PI 3-kinase blocks BMP-2-induced alkaline phosphatase expression.** *A*, 2T3 cells were incubated with 12.5 μM Ly-294002 before treatment with BMP-2. Cell lysates were immunoprecipitated with anti-phosphotyrosine antibody followed by PI 3-kinase assay. *Arrow* indicates the position of PI 3-phosphate. *B*, 2T3 cells were treated with ME_2SO vehicle or Ly-294002 prior to incubation with BMP-2 for 48 h. After fixation with formalin, the cells were stained with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium for alkaline phosphatase as described (20). *C*, lysates from 2T3 cells incubated with Ly-294002 and BMP-2 were assayed for alkaline phosphatase activity using pNPP as substrate as described (32). Mean ± S.E. of triplicate measurements is shown. *D*, 2T3 cells were transfected with vector alone or pSRaΔp85 expressing the DN p85 subunit of PI 3-kinase. At 24-h post-transfection, the cells were treated with BMP-2 for 48 h and stained for alkaline phosphatase (20). Cells in *panels a* and *c* were incubated in the absence of BMP-2, and cells in *panels b* and *d* in the presence of BMP-2. *E*, lysates form dominant-negative PI 3-kinase transfected 2T3 cells, which were incubated with or without BMP-2, were assayed for alkaline phosphatase activity as described (32).

cells were incubated with BMP-2 for different periods of time, and the lysates were immunoprecipitated with an antibody that recognizes the regulatory p85 subunit of PI 3-kinase. Immunocomplex PI 3-kinase assay showed that BMP-2 moderately increased PI 3-kinase activity in a time-dependent manner (Fig. 1*A*). The mechanism by which PI 3-kinase is activated by growth factors depends upon tyrosine phosphorylation of protein(s) where the SH2 domain of the regulatory p85 subunit of PI 3-kinase associates with specific phosphotyrosine (15). Therefore, we first tested the effect of BMP-2 on tyrosine phosphorylation of protein in 2T3 cells. Immunoblotting of BMP-2-treated 2T3 cell lysates showed increased tyrosine phosphorylation of multiple proteins to a variable extent (Fig. 1*B*). Although the regulatory p85 subunit of PI 3-kinase has been reported to be tyrosine-phosphorylated, BMP-2 did not induce tyrosine phosphorylation of any 85-kDa protein (Fig. 1*B*). However, anti-phosphotyrosine immunoblotting of p85 immunoprecipitates from BMP-2-treated 2T3 cell lysate showed increased association of the tyrosine-phosphorylated protein with the p85 regulatory subunit of PI 3-kinase (Fig. 1*C*). These data suggest that PI 3-kinase is present in a tyrosine-phosphorylated signaling complex in BMP-2-treated cells indicating a possibility where the lipid kinase can be activated by association with tyrosine-phosphorylated proteins in response to BMP-2. Anti-phosphotyrosine antibody was therefore used to immunoprecipitate and assay PI 3-kinase from lysates of BMP-2-treated 2T3 cells. BMP-2 significantly increased anti-phosphotyrosine-associated PI 3-kinase activity in a time-dependent manner (Fig. 1*D*) similar to that demonstrated in PI 3-kinase immunoprecipitates. Together these data indicate that BMP-2 regulates PI 3-kinase activity by stimulating association of p85 subunit of PI 3-kinase with a tyrosine-phosphorylated protein present in a signaling complex.

PI 3-Kinase Regulates BMP-2-induced Alkaline Phosphatase Production—We showed previously that BMP-2-induced mature osteoblast formation in 2T3 cells is associated with expression of alkaline phosphatase mRNA, which directly correlates with its enzymatic activity (3, 20). Therefore, we used alkaline phosphatase activity as a measure of osteoblast differentiation. To study the potential role of PI 3-kinase in osteoblast differentiation, we used a pharmacological inhibitor of PI 3-kinase, Ly-294002. 2T3 cells were treated with Ly-294002 followed by incubation with BMP-2. PI 3-kinase assay of anti-phosphotyrosine immunoprecipitates showed that Ly-294002 blocked

BMP-2-induced PI 3-kinase activity (Fig. 2*A*, compare *lane 4* with *lane 2*). To test the effect of PI 3-kinase on BMP-2-mediated alkaline phosphatase expression, we treated 2T3 cells with BMP-2 in the presence and absence of Ly-294002 and stained for alkaline phosphatase activity. As expected, BMP-2 increased expression of alkaline phosphatase (Fig. 2*B*, compare *panel b* with *panel a*). Inhibition of PI 3-kinase activity by Ly-294002 significantly blocked BMP-2-induced alkaline phosphatase (Fig. 2*B*, compare *panel d* with *panel b*). In a parallel experiment, we assayed alkaline phosphatase activity in the lysates of 2T3 cells incubated with Ly-294002 plus BMP-2 and with BMP-2 alone. Inhibition of PI 3-kinase activity completely blocked BMP-2-induced alkaline phosphatase activity (Fig. 2*C*). To further confirm the requirement of PI 3-kinase in this process, we transfected 2T3 cells with a dominant-negative p85 regulatory subunit that blocks the enzymatic activity of the catalytic p110 subunit of PI 3-kinase (34). The cells were then treated with BMP-2, and expression of alkaline phosphatase was tested by activity staining. Expression of dominant-negative PI 3-kinase significantly blocked BMP-2-induced alkaline phosphatase expression (Fig. 2*D*, compare *panel d* with *panel b*). Similarly, expression of dominant-negative p85 subunit of PI 3-kinase significantly blocked alkaline phosphatase activity in the lysates of 2T3 cells (Fig. 2*E*). These data indicate that PI 3-kinase-dependent signal transduction regulates BMP-2-induced expression of alkaline phosphatase.

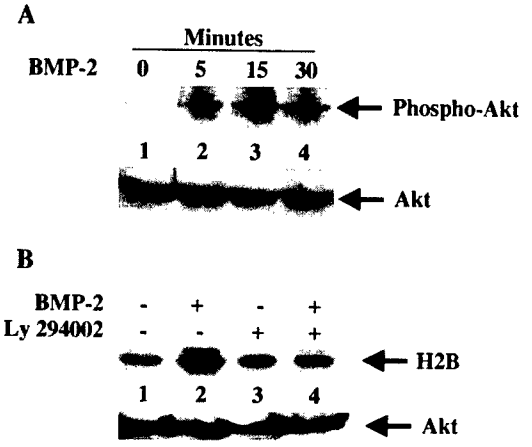

FIG. 3. *A*, BMP-2 increases Akt serine/threonine kinase activity. Serum-deprived 2T3 cells were incubated with BMP-2 for indicated periods of time. 50 μg of cleared cell lysates were immunoblotted with phospho-Akt antibody, which is specific for activation state of Akt. *Bottom panel* shows immunoblot analysis of the same samples with Akt antibody. *B*, effect of Ly-294002 on BMP-2-induced Akt activity in 2T3 cells. Serum-deprived 2T3 cells were treated with 12.5 μM Ly-294002 for 1 h before incubation with BMP-2. Cleared cell lysates (100 μg) were immunoprecipitated with Akt antibody followed by immunocomplex kinase assay using histone H2B as substrate in the presence of [γ-^{32}P]ATP as described under "Experimental Procedures." *Bottom panel* shows immunoblot analysis of the same samples with Akt antibody.

BMP-2 Stimulates Akt Serine/Threonine Kinase—Although PI 3-kinase-independent activation of Akt has been documented, one of the principal downstream targets of PI 3-kinase is the serine/threonine kinase Akt (35, 36). Phosphorylation of Akt by 3-phosphoinositide-dependent kinases 1 and 2 represents the activated state of this enzyme (37). To investigate whether BMP-2-induced osteoblast differentiation is mediated by Akt, we investigated the effect of BMP-2 on activation of Akt in 2T3 cells. Activation was determined by immunoblot analysis of lysates from BMP-2-treated 2T3 cells using an anti-phospho-Akt-specific antibody that recognizes the activated form. Akt was activated in response to BMP-2 in a time-dependent manner (Fig. 3*A*). The kinetics of BMP-2-induced Akt activation were similar to those of BMP-2-induced PI 3-kinase activity in these cells (Fig. 1), suggesting that activation of Akt may be PI 3-kinase-dependent. To test this directly, we treated 2T3 cells with Ly-294002 followed by BMP-2. Similar to Akt activation determined by anti-phospho-Akt antibody (Fig. 3*A*), BMP-2 stimulated Akt kinase activity as determined by Akt immunocomplex kinase assay using histone H2B as substrate (Fig. 3*B*). Inhibition of PI 3-kinase by Ly-294002 abolished BMP-2-induced Akt activity (Fig. 3*B*, compare *lane 4* with *lane 2*). These data indicate that BMP-2 stimulates Akt kinase via activation of PI 3-kinase.

Akt Regulates BMP-2-induced Alkaline Phosphatase Expression—Because PI 3-kinase is required for BMP-2-induced alkaline phosphatase activity (Fig. 2), we investigated whether the downstream target of PI 3-kinase, Akt serine/threonine kinase, regulates this process. We transfected a kinase-deficient Akt, which this group and others (25, 36) have shown previously to act in a dominant-negative fashion, into 2T3 cells. Transfected cells were then incubated with BMP-2, and expression of alkaline phosphatase was tested by activity staining. Expression of dominant-negative Akt significantly blocked

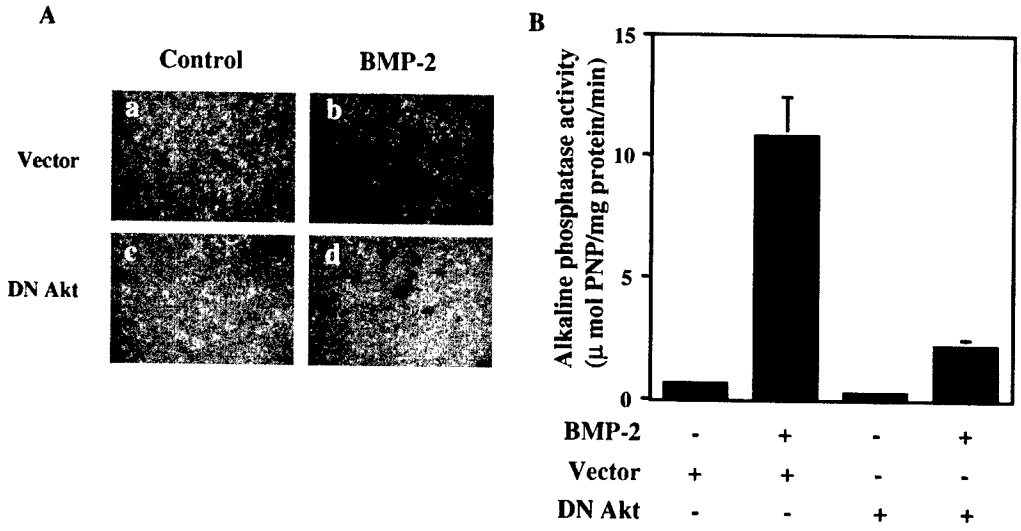

FIG. 4. **Akt regulates BMP-2-induced alkaline phosphatase activity.** *A*, 2T3 cells were transfected with vector (*panels a* and *b*) or pCMV6-HA-Akt (K179M) expressing DN-Akt (*panels c* and *d*). Transfected cells were incubated with BMP-2. 48 h post-incubation, the cells were stained for alkaline phosphatase expression (20). *B*, lysates from vector or dominant-negative Akt-transfected 2T3 cells, which were incubated with BMP-2, were assayed for alkaline phosphatase activity (32).

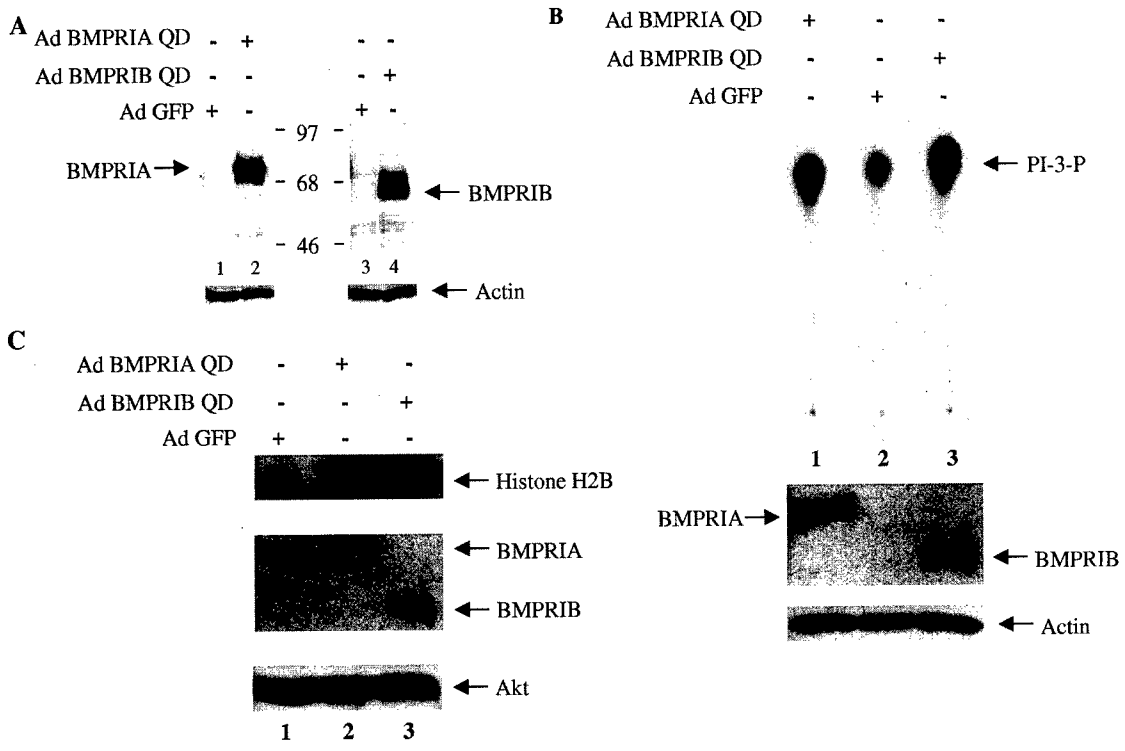

FIG. 5. **BMPRIA and BMPRIB activate PI 3-kinase and Akt in 2T3 cells.** *A*, 2T3 cells were infected with a control Ad-GFP or Ad-BMPRIA-QD or Ad-BMPRIB-QD as described under "Experimental Procedures." Equal amounts of cleared cell lysate were immunoblotted with anti-HA antibody to detect expression of the receptor proteins. *Arrow* indicates the position of BMPRIA and BMPRIB, respectively. *Bottom panels* demonstrate immunoblotting of the same lysate with actin antibody. *B*, quiescent 2T3 cells were infected with the indicated adenovirus vectors. Six hours post-infection, 100 μg of cell lysates were immunoprecipitated with anti-phosphotyrosine antibody followed by PI 3-kinase assay as described under "Experimental Procedures." *Arrow* indicates the position of PI-3-P. *Middle* and *bottom panels* show immunoblot analyses of the same lysates with anti-HA and anti-actin antibodies, respectively. *C*, 50 μg of lysates from *panel B* were immunoprecipitated with Akt followed by immunocomplex kinase assay using histone H2B as substrate as described in Fig. 3*B*. *Arrow* in the *top panel* shows phosphorylated histone H2B. *Middle* and *bottom panels* show anti-HA and anti-Akt immunoblotting of the same samples, respectively.

BMP-2-induced alkaline phosphatase staining (Fig. 4*A*, compare *panel d* with *panel b*). To confirm this observation, the alkaline phosphatase activity in the lysates was measured. Expression of dominant-negative Akt inhibited alkaline phosphatase activity (Fig. 4*B*). Together these data indicate that BMP-2-stimulated PI 3-kinase/Akt signaling cascade regulates alkaline phosphatase, an early marker of osteoblast differentiation.

BMPRIA and BMPRIB Regulate PI 3-Kinase and Akt Activities—In the activated oligomeric receptor complex, BMPRI acts as the signal transmitter (6, 38). Differential action of BMPRIA and BMPRIB has been reported during vertebrate embryonic development and chick limb bud development (39, 40). To examine whether PI 3-kinase and Akt are stimulated by both these receptors, we used adenovirus vectors containing constitutively active BMPRIA (Ad-BMPRIA-QD) and BMPRIB (Ad-BMPRIB-QD). Infection of 2T3 cells with these vectors showed significant levels of expression of both these receptors (Fig. 5*A*). As expected, BMPRIA migrated slightly slower than did BMPRIB (Fig. 5*A*) (41, 42). Constitutively active receptor expression was detectable as early as 6 h of vector infection (Fig. 5, *B* and *C*). Therefore, we used anti-phosphotyrosine immunoprecipitates from these adenovirus vector-infected 2T3 cells for PI 3-kinase activity. Expression of constitutively active BMPRIA and BMPRIB significantly activated PI 3-kinase activity, respectively (Fig. 5*B*, *top panel*, compare *lanes 1* and *3* with *lane 2*, respectively). Because Akt is downstream of BMP-2-stimulated PI 3-kinase (Fig. 3), immunocomplex kinase assay of Akt immunoprecipitates from lysate of Ad-BMPRIA-QD- and Ad-BMPRIB-QD-infected 2T3 cells was performed. Both BMPRIA and BMPRIB significantly increased Akt kinase activity (Fig. 5*C*, *top panel*, compare *lanes 2* and *3* with *lane 1*). These data indicate that in osteoblasts both type I receptors are capable of contributing in activation of PI 3-kinase/Akt signaling.

PI 3-Kinase and Akt Regulate BMP-2-induced BMP-2 Transcription—We previously reported that BMP-2 stimulates *BMP-2* gene expression by increased transcription during osteoblast differentiation (3, 20, 33). This autoregulation of BMP-2 expression maintains its sustained effect on terminally differentiated osteoblasts. Because mechanisms of signaling of BMP-2 transcription are not known, we investigated the role of PI 3-kinase in *BMP-2* gene transcription. A reporter construct in which the *BMP-2* promoter drives firefly luciferase cDNA (BMP-2-LUC) was transfected either with dominant-negative p85 subunit of PI 3-kinase or with kinase-dead Akt into 2T3 cells. Incubation of transiently transfected cells with BMP-2 increased the reporter gene expression indicating that BMP-2 autoregulates its own transcription (Fig. 6, *A* and *B*). However, transfection of dominant-negative PI 3-kinase or dominant-negative Akt significantly blocked BMP-2-induced BMP-2 transcription (Fig. 6, *A* and *B*, respectively). These data indicate that PI 3-kinase/Akt signaling regulates *BMP-2* gene transcription induced by BMP-2.

All the known biological activities of BMP-2 are mediated by BMP-specific Smad proteins (4, 7). BMP-activated Smad, such as Smad5, stimulates transcription of target genes required for osteoblast differentiation. Because BMP-2 stimulates transcription of the *BMP-2* gene (20, 33), we investigated the effect of Smad5 on *BMP-2* promoter activity in 2T3 cells using the BMP-2-LUC reporter construct. Transfection of Smad5 alone increased *BMP-2* gene transcription in these cells in a ligand-independent manner (Fig. 6, *C* and *D*). Transfection of dominant-negative PI 3-kinase blocked Smad5-induced transcrip-

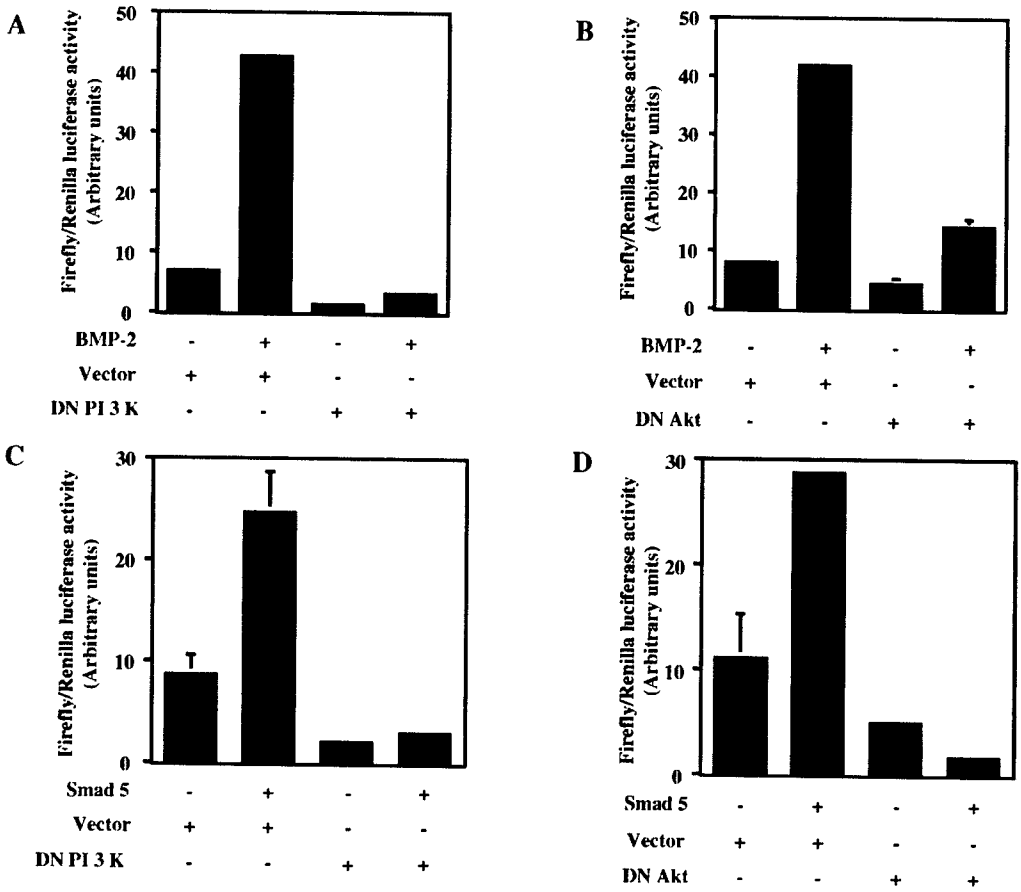

FIG. 6. **PI 3 kinase signaling regulates transcription of BMP-2.** *A* and *B*, PI 3-kinase and Akt regulate *BMP-2* gene transcription. BMP-2-LUC reporter plasmid was cotransfected with vector or dominant-negative p85 construct (*panel A*) or dominant-negative Akt expression construct (*panel B*) into 2T3 cells. Transiently transfected cells were treated with BMP-2. Luciferase activity was determined as a measure of transcriptional activation as described under "Experimental Procedures." *C* and *D*, PI 3-kinase and Akt kinase regulate Smad5-dependent *BMP-2* gene transcription. BMP-2-LUC reporter plasmid was cotransfected with Smad5 expression vector alone or along with a dominant-negative construct of p85 (*panel C*) or dominant-negative Akt (*panel D*) into 2T3 cells. Luciferase activity was determined as described under "Material and Methods." Mean ± S.E. of triplicate measurements is shown.

tion of *BMP-2* promoter-driven reporter gene (Fig. 6*C*). Similarly transfection of dominant-negative Akt also inhibited Smad5-dependent transcription of BMP-2 promoter (Fig. 6*D*). These data provide evidence that PI 3-kinase/Akt signal transduction pathway integrates into the nucleus to influence Smad-mediated transcription.

Next we investigated the mechanism by which Akt may regulate BMP-2-induced Smad-dependent transcription. Analysis of the primary amino acid sequence of Smad5 and Smad1 revealed absence of any consensus Akt phosphorylation site (RXRXX(S/T)). The mechanism by which BMP regulates Smad function is by inducing translocation of these cytosolic proteins into the nucleus leading to transcription of target genes (4, 5, 7). To examine whether Akt regulates Smad translocation, we infected 2T3 cells with Ad-DN-Akt, an adenovirus vector containing HA-tagged dominant-negative Akt, followed by incubation with BMP-2. Translocation of BMP-specific Smad was studied by immunofluorescence using an antibody that recognizes both Smad5 and Smad1. As expected BMP-2 stimulated translocation of cytosolic and perinuclear Smad into the nucleus with punctate appearance on a diffused nuclear staining (Fig. 7, compare *panel B* with *panel A*) (43). Expression of dominant-negative Akt significantly blocked BMP-2-induced nuclear localization of Smad (Fig. 7, compare *panel D* with *panel B*). These data indicate that Akt, in the absence of its consensus phosphorylation site, regulates translocation of BMP-specific Smads into the nucleus, and hence indirectly modulates Smad-dependent transcription.

DISCUSSION

These studies represent the first demonstration of activation of the PI 3-kinase/Akt pathway in response to the osteogenic factor BMP-2. We demonstrate that BMP-2-induced expression of alkaline phosphatase, an enzyme expressed during osteoblast differentiation of progenitor cells into mature osteoblast, requires activation of PI 3-kinase and its downstream target Akt serine/threonine kinase. Our data also provide the first evidence that PI 3-kinase and Akt modulate autoregulation of *BMP-2* gene transcription and that a cross-talk exists between the BMP-specific Smad and PI 3-kinase/Akt signaling pathway for regulation of BMP-2 transcription.

Activation of PI 3-kinase regulates cellular processes including proliferation, migration, secretion, endocytosis, and protein transport (17). Recently a role of PI 3-kinase has been shown in TGF-β-induced epithelial and endothelial cell survival and epithelial to mesenchymal transition (19, 44, 45) indicating that activation of serine/threonine kinase receptor utilizes this central lipid kinase as one of the signaling mechanisms similar to receptor tyrosine kinases. Recently PI 3-kinase has been implicated in myogenic differentiation (13, 46, 47). Also insulin-induced adipocyte differentiation utilizes activation of PI 3-kinase pathway (48, 49). We have shown here that the osteogenic differentiation factor BMP-2 stimulates PI 3-kinase activity (Fig. 1). Increased PI 3-kinase activity is often associated with receptor and non-receptor tyrosine kinase-mediated signal transduction in which activation of this lipid kinase is a result

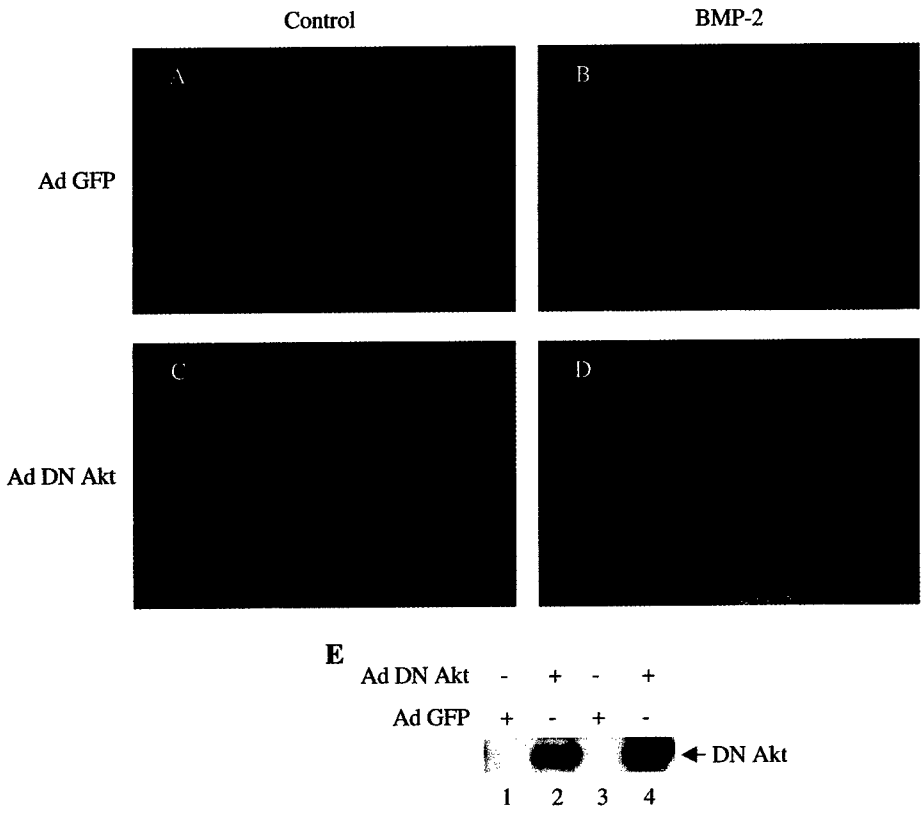

FIG. 7. **Expression of dominant-negative Akt blocks BMP-2-induced nuclear translocation of BMP-specific Smad.** 2T3 cells in chamber slides were infected with 100 m.o.i. Ad-GFP (*panels A* and *B*) or Ad-DN-Akt (*panels C* and *D*) for 24 h before incubation with BMP-2 as described under "Experimental Procedures." The cells were stained with anti-Smad5/1 antibody as described under "Experimental Procedures." *Panel E* shows immunoblot analysis of adenovirus-infected cell lysates with anti-HA antibody to demonstrate expression of dominant-negative Akt.

of its association with specific tyrosine-phosphorylated proteins (15). In this report we demonstrate that activation of the BMP receptor results in increased tyrosine phosphorylation of proteins leading to association of the regulatory subunit of PI 3-kinase with tyrosine-phosphorylated proteins (Fig. 1, *B* and *C*). Also, we document BMP-2 induced PI 3-kinase activity in the tyrosine-phosphorylated protein fraction (Fig. 1*D*). Additionally we demonstrate that both BMPRIA and BMPRIB are involved in activation of PI 3-kinase in the tyrosine-phosphorylated protein fraction (Fig. 5). However, we were unable to detect any association of PI 3-kinase with the BMPRI in the BMPRI immunoprecipitates (data not shown). These data indicate that not only receptor tyrosine kinases, but serine/threonine kinase receptor utilizes the similar tyrosine phosphorylation mechanism to stimulate PI 3-kinase activity. It is evident from our data that the PI 3-kinase activity in p85 immunoprecipitates is somewhat less (Fig. 1*A*) than that in anti-phosphotyrosine immunoprecipitates (Fig. 1*D*). One reason for this may be that p85 antibody recognizes the total pool of PI 3-kinase present in the cell whereas anti-phosphotyrosine antibody recognizes only the activated form of PI 3-kinase, which is associated with tyrosin-phosphorylated proteins in response to BMP-2.

Differentiation of cells is always associated with induction of specific protein markers resulting from cell- and differentiation-specific transcriptional programs. Thus a hallmark of osteoblast differentiation is expression of alkaline phosphatase, which is induced early in the differentiation process (3, 20, 32). We showed previously that BMP-2 stimulated alkaline phosphatase mRNA and protein expression in 2T3 cells during the initiation of differentiation (3, 20). Using a PI 3-kinase inhibitor, we have now demonstrated that activation of this lipid kinase is essential for BMP-2-induced alkaline phosphatase activity in these cells (Fig. 2, *A–C*). Furthermore the absence of alkaline phosphatase activity in 2T3 cells expressing a dominant-negative PI 3-kinase conclusively establishes the requirement of PI 3-kinase for this osteoblast differentiation-specific early enzyme activity (Fig. 2, *D* and *E*). In addition, in long term culture, incubation of 2T3 cells with PI 3-kinase inhibitor Ly-294002 in the presence of BMP-2 completely blocked mineralized bone nodule formation indicating further the importance of this lipid kinase in osteoblast differentiation (data not shown).

Many biological functions of PI 3-kinase are regulated by its direct downstream target, Akt serine/threonine kinase (16, 25, 35). The products of PI 3-kinase, the D_3-phosphoinositides, bind to the N-terminal plextrin homology (PH) domain of Akt to recruit it into the plasma membrane where PDK1 and PDK2 phosphorylate it, resulting in its full activation (37, 50). Although a diverse group of proteins that function in many different cellular processes has been identified as substrates of Akt, the role of Akt in inhibition of apoptosis has been most extensively studied (35). This action of Akt is mediated by phosphorylation of pro-apoptotic protein BAD and caspase 9 (51, 52). Terminal cellular differentiation is a post-mitotic phenomenon where cells acquire an apoptosis-resistant phenotype (53). Recently Akt has been implicated in myogenic differentiation (54, 55). Also Akt regulates IGF-induced myotube formation, as a downstream target of PI 3-kinase. This action of Akt is mediated by phosphorylation of Raf-1, which negatively regulates its kinase activity and results in maintenance of highly differentiated myotubes (56). Here we show that the osteogenic factor BMP-2 stimulates Akt activity in a PI 3-kinase-dependent manner in 2T3 osteoblast precursor cells in response to BMP-2 (Fig. 3). Expression of dominant-negative Akt resulted in significant inhibition of BMP-2-induced alkaline phosphatase activity in these cells, indicating that Akt regulates the expression of this early osteoblast-specific marker protein (Fig. 4). Along with 2T3 cells, which we established and used extensively as a model for osteoblast growth and differentiation (3, 20–23), we used another multipotent cell line, C2C12, which has the potential to undergo osteoblast differentiation in the presence of BMP-2 (24, 57). Similar to 2T3 cells, BMP-2 stimulated Akt kinase activity in these cells, and expression of

dominant-negative Akt significantly blocked BMP-2-induced alkaline phosphatase activity (data not shown). These results further confirm our observation that PI 3-kinase/Akt signaling cascade regulates osteoblast differentiation in cells, which have the potential to undergo differentiation to osteoblasts in response to BMP-2.

BMP-regulated Smad1 or Smad5 after dimerization with Smad4 binds to specific DNA elements present in the target genes (4, 5). Smads also interact with other transcription factors, transcriptional coactivators, and cell-specific DNA-binding proteins to specify transcriptional programs that determine the biological functions of BMPs (4, 5, 58). C-terminal phosphorylation of Smad by the type I BMP receptor is sufficient for its translocation to the nucleus, DNA binding, and stimulation of transcription (4, 5, 7, 43). Here we demonstrate that Smad5 and Smad1 stimulate BMP-2 transcription in 2T3 and C2C12 progenitor cells (Fig. 6 and data not shown).

Expression of Smad proteins has been shown to induce transcription of target gene in a ligand-independent manner and partially mimics the action of the ligands (59, 60). However, the precise mechanism by which different signaling inputs regulate Smad-dependent transcription has not been extensively studied. For example ERK1/2-dependent serine phosphorylation of Smad1 in the linker domain has been shown to block its translocation to the nucleus and transcriptional activity, indicating a negative regulation of this transcription factor by this kinase (61). We have provided evidence here that the PI 3-kinase/Akt signaling cascade regulates Smad5-dependent BMP-2 transcription (Fig. 6). Although BMP-specific Smad5 and Smad1 do not contain any Akt phosphorylation site, we demonstrate that Akt regulates translocation of Smad5 and Smad1 into the nucleus (Fig. 7). Because Smad-dependent transcription is dependent upon its ability to be localized in the nucleus (43), our observation that Akt regulates Smad translocation provides a mechanism by which Akt modulates BMP-2-stimulated Smad-dependent transcription. These results represent the first demonstration of a signal transduction pathway that positively regulates Smad-dependent transcription of *BMP-2* gene. Furthermore, our data indicate that interplay between the PI 3-kinase/Akt signaling cascade and BMP-specific Smad-induced transcription of lineage-specific genes regulates osteoblastogenesis.

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Phosphatidylinositol 3-Kinase Regulates Bone Morphogenetic Protein-2 (BMP-2)-induced Myocyte Enhancer Factor 2A-dependent Transcription of BMP-2 Gene in Cardiomyocyte Precursor Cells*[S]

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The growth and differentiation factor bone morphogenetic protein-2 (BMP-2) regulates cardiac development during vertebrate embryogenesis. In cardiac precursor cells, BMP-2 has recently been shown to induce expression of cardiac transcription factors, including myocyte enhancer factor 2A (MEF-2A). The specific signal transduction mechanism by which BMP-2 regulates these actions is not known. We investigated the role of phosphatidylinositol (PI) 3-kinase in regulating these processes in cardiomyocyte precursor CL6 cells. BMP-2 increased PI 3-kinase activity in these cells in a time-dependent manner, resulting in increased expression of sarcomeric myosin heavy chain (MHC) and MEF-2A. Inhibition of PI 3-kinase abolished these actions of BMP-2, indicating the involvement of PI 3-kinase in these processes. Furthermore, BMP-2 stimulated specific protein·DNA complex formation when an MEF-2 DNA recognition element was used as probe. Antibody supershift assay confirmed the presence of MEF-2A in this protein·DNA complex. Inhibition of PI 3-kinase activity completely prevented the MEF-2A·DNA complex formation. BMP-2 also increased transcription of a reporter gene driven by an MEF-2-specific DNA element in a PI 3-kinase-dependent manner. Ectopic expression of MEF-2A increased BMP-2 transcription to the same extent induced by BMP-2, indicating that MEF-2A may participate in BMP-2 autoregulation in CL6 cells. Expression of dominant negative PI 3-kinase completely abolished BMP-2-induced as well as MEF-2A-mediated BMP-2 transcription. Furthermore expression of MEF-2A increased MHC expression in a PI 3-kinase-dependent manner. Together these data provide the first evidence that BMP-2-induced PI 3-kinase signaling regulates MEF-2A expression and define a mechanism of MEF-2A-dependent BMP-2 transcription.

Bone morphogenetic proteins were originally identified as growth and differentiation factors for osteogenic cells and are now considered as multifunctional polypeptides (1). BMPs[1] play important roles in the development of many organs, including lung, kidney, gut, skin, teeth, and heart (2). BMP-2, a member of this family of proteins, is expressed during mouse embryonic heart development (3). A similar expression pattern of BMP-2 is conserved in *Xenopus*, where this growth and differentiation factor is expressed in the heart progenitor cells and in the mature heart (4). During chick embryogenesis, the tissues that express BMP-2 are in contact with the precursor cells committed to cardiac muscle lineage. Furthermore, introduction of BMP-2 *in vivo* induces expression of cardiac-specific transcription factors (5). Finally, abnormal heart development leads to embryonic lethality in BMP-2 null mice (6).

The MEF-2 family of transcription factors has been shown to play a pivotal role in myogenesis of smooth muscle, skeletal, and cardiac cells (7). MEF-2 DNA binding elements are present in many cardiac muscle genes. MEF-2A, MEF-2C, and MEF-2D bind the consensus DNA element with the same specificity, whereas MEF-2B binds with relatively reduced affinity (8). Addition of noggin, a BMP-2 antagonist, to chick embryo explants induced loss of cardiac-specific transcription factors, including MEF-2A (9). One of the major kinases that regulate MEF-2 transcriptional activity is the p38 mitogen-activated protein kinase (10). More recently, PI 3-kinase, which is activated by the receptor and nonreceptor tyrosine kinases, has been shown to regulate MEF-2 transcriptional activity and myogenesis (11, 12). Thus PI 3-kinase is required for insulin-like growth factor-1 receptor tyrosine kinase-induced myogenesis in culture and during mouse embryogenesis (13, 14).

BMP-2 acts as a survival factor for neonatal cardiac myocytes via Smad 1, the downstream target of BMP receptor (15). BMP-2 also induces expression of cardiac transcription factors in embryonic teratocarcinoma cells (16). The mechanism by which BMP-2 regulates cardiac development and cardiac gene expression is largely unknown. Here we show that BMP-2 activates PI 3-kinase in P19CL6 embryonic carcinoma cells, which differentiate along the cardiomyocyte lineage. BMP-2-induced expression and DNA binding of MEF-2 transcription factor are dependent upon PI 3-kinase activity. In addition we show that BMP-2-induced PI 3-kinase activity regulates MEF-2-dependent reporter gene transcription. Finally, we demon-

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[1] The abbreviations used are: BMPs, bone morphogenetic proteins; MEF, myocyte enhancer factor; PI, phosphatidylinositol; MHC, myosin heavy chain; EMSA, electrophoretic mobility shift assay; PKC, protein kinase C.

strate that BMP-2-induced BMP-2 gene transcription is regulated by MEF-2 in a PI 3-kinase-dependent manner.

EXPERIMENTAL PROCEDURES

Materials—Recombinant BMP-2 was obtained from Genetics Institute, Cambridge, MA. Na_3VO_4, Nonidet P-40, and phenylmethylsulfonyl fluoride were purchased from Sigma. Aprotinin was obtained from Bayer. Antibody against the p85 and p110 subunits of PI 3-kinase, MEF-2A, and noggin were obtained from Santa Cruz Biotechnology. Anti-phosphotyrosine antibody was purchased from Upstate Biotechnology Inc. MF-20 antibody recognizing sarcomeric MHC was obtained from the Developmental Studies Hybridoma Bank, University of Iowa. Tissue culture reagents and LipofectAMINE were obtained from Invitrogen. A dual luciferase assay kit was purchased from Promega Inc. Nuclear fraction extraction reagents were obtained from Pierce. The pSR$\alpha\Delta$p85 plasmid encoding for a dominant negative regulatory subunit for PI 3-kinase and Ad Myr-p110 adenovirus vector expressing the constitutively active p110 catalytic subunit of PI 3-kinase were gifts from Dr. Wataru Ogawa, Kobe University, Japan. The MEF-2A expression plasmid was obtained from Dr. Richard Prywes (Columbia University, New York).

Cell Culture—P19CL6 cells, described as CL6 in this report, and CL6-Noggin cells expressing noggin were kind gifts of Dr. I. Komuro, Department of Cardiovascular Medicine, University of Tokyo, Japan. CL6 cells undergo differentiation in the presence of 1% Me_2SO into beating cardiomyocyte and express cardiac-specific genes (16). The cells were grown in α-minimal essential medium with 10% fetal bovine serum. The cells were serum-deprived for 24 h to make them quiescent. To induce MEF-2A expression, the cells were grown in 1% Me_2SO or in the indicated concentration of BMP-2.

Adenovirus Infection—CL6 cells were infected with a multiplicity of infection of 50 for Ad Myr-p110, essentially as described before (17).

Immunofluorescence—Immunofluorescence detection of sarcomeric MHC was performed essentially as described previously (17). Briefly, treated cells in eight-well chamber slides were fixed in cold methanol for 15 min followed by blocking with donkey IgG for 15 min. The cells were then incubated with MF-20 for 30 min. Stained cells were treated with Cy-tagged secondary anti-mouse antibody for 30 min. The cells were visualized using a confocal microscope (Olympus FluoView 500) and photographed using FluoView software.

Immunoprecipitation, Immunoblotting, and PI 3-kinase Assay—Cells were lysed in radioimmune precipitation assay buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 0.05% aprotinin, and 1% Nonidet P-40). Cleared cell lysates were prepared by centrifugation at 10,000 × *g* for 30 min at 4 °C. Protein concentration was determined in the lysate, and an equal amount of protein was immunoprecipitated with the p85 regulatory subunit of PI 3-kinase or anti-phosphotyrosine antibody. The immunoprecipitates were used in the PI 3-kinase assay using PI as substrate in the presence of [γ-^{32}P]ATP, and the products were separated by thin layer chromatography followed by autoradiography (18, 19). Immunoblotting of the lysates was performed using appropriate antibodies as described previously (20–23).

Electrophoretic Mobility Shift Assay—CL6 cells were incubated with BMP-2 in the presence and absence of Ly294002. Nuclear extracts were prepared using a kit according to the method provided by the vendor. An MEF-2 DNA probe was prepared by annealing the oligonucleotide 5′-GATCGCTCTAAAAATAACCCTGTCG-3′ with its complementary strand and labeling the double-stranded oligonucleotide consensus sequence using [γ-^{32}P]ATP and T4 polynucleotide kinase (24). The EMSA was performed using 10 μg of the nuclear extract as described previously (25, 27). To determine the specificity of protein·DNA interaction, the nuclear extract was incubated with a 100-fold excess of cold double-stranded oligonucleotide and incubated farther with the labeled probe. For supershift analysis, the nuclear extracts were incubated with the MEF-2A or cyclin D1 antibody on ice for 30 min before binding reaction was performed as described before (25–27).

Transfection and Luciferase Assay—The BMP-2-LUC reporter plasmid, in which the firefly luciferase gene is driven by a 2.7-kb 5′-flanking sequence of BMP-2 gene, has been described before (17, 28, 29). BMP-2-LUC reporter plasmid was cotransfected with different expression plasmids using LipofectAMINE Plus reagent as described (20, 21, 26, 30). A cytomegalovirus promoter-driven *Renilla* luciferase plasmid was included in the transfection mix to correct for transfection efficiency. Luciferase activity was determined using a dual luciferase assay kit.

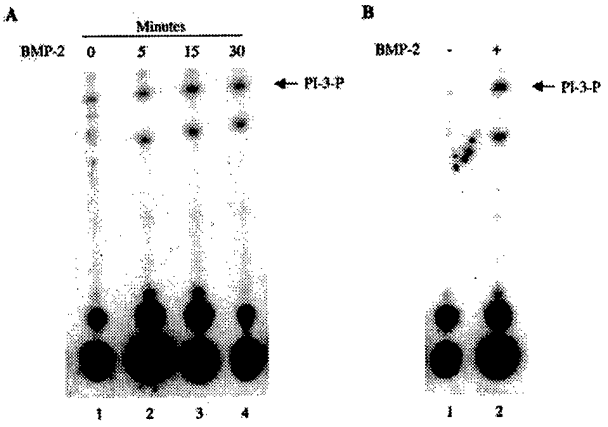

FIG. 1. **Effect of BMP-2 on PI 3-kinase activity in CL6 cardiomyocyte precursor cells.** *A*, serum-deprived CL6 cells were incubated with 100 ng/ml BMP-2 for the indicated periods of time. Equal amounts of cleared cell lysates were immunoprecipitated with anti-p85 regulatory subunit antibody of PI 3-kinase, followed by immune complex kinase assay as described under "Experimental Procedures." The 3-phosphoinositide was separated by TLC. *B*, the cells were stimulated with BMP-2 for 15 min. PI 3-kinase assay was performed in anti-phosphotyrosine immunoprecipitates and analyzed by TLC. *Arrows* indicate the position of PI 3-phosphate.

RESULTS

BMP-2 Stimulates PI 3-kinase Activity—CL6 cells, a clonal derivative of embryonic P19 teratocarcinoma cells, undergo differentiation into beating cardiomyocytes when treated with Me_2SO in a BMP-2-dependent manner (16). To investigate the role of PI 3-kinase in response to BMP-2, CL6 cells were incubated with 100 ng/ml BMP-2 for different periods of time. The lysates were then immunoprecipitated with an antibody recognizing the p85 regulatory subunit of PI 3-kinase. The immunoprecipitates were used in immunocomplex PI 3-kinase assays. BMP-2 increased PI 3-kinase activity in a time-dependent manner (Fig. 1*A*). The mechanism by which PI 3-kinase is activated by receptor and nonreceptor tyrosine kinases is by direct association of the SH-2 domain of the regulatory subunit with the phosphotyrosine of the proteins (31). Therefore, we assayed PI 3-kinase activity in the anti-phosphotyrosine immunoprecipitates from BMP-2-treated CL6 cells. BMP-2 significantly increased PI 3-kinase activity in the anti-phosphotyrosine immunoprecipitates (Fig. 1*B*). These data indicate that activation of BMP receptor serine threonine kinase in CL6 cells activated PI 3-kinase in the tyrosine-phosphorylated protein fraction.

BMP-2-induced PI 3-kinase Is Required for Cardiomyocyte Differentiation—To examine the role of PI 3-kinase in BMP-2-induced cardiomyocyte differentiation, we used Ly294002, a pharmacological inhibitor of PI 3-kinase. Incubation of CL6 cell with this inhibitor completely blocked BMP-2-induced PI 3-kinase activity (Fig. 2*A*). Mature cardiomyocytes express sarcomeric MHC. During differentiation of CL6 cells to cardiomyocytes, MHC is highly expressed and serves as a marker for mature cardiomyocytes (16). To test the effect of PI 3-kinase on BMP-2-induced cardiomyocyte differentiation, CL6 cells were incubated with Ly294002 in the presence and absence of BMP-2. The cells were stained with anti-MHC antibody MF-20. As expected, BMP-2 efficiently stimulated expression of MHC, suggesting induction of mature cardiomyocytes (Fig. 2*B*, compare *panel b* with *panel a*). Inhibition of PI 3-kinase activity by Ly294002 abolished BMP-2-induced MHC expression (Fig. 2*B*, compare *panel d* with *panel b*). To further confirm the involvement of PI 3-kinase, we used an adenovirus vector (Ad Myr-p110) containing the constitutively active myristoylated p110

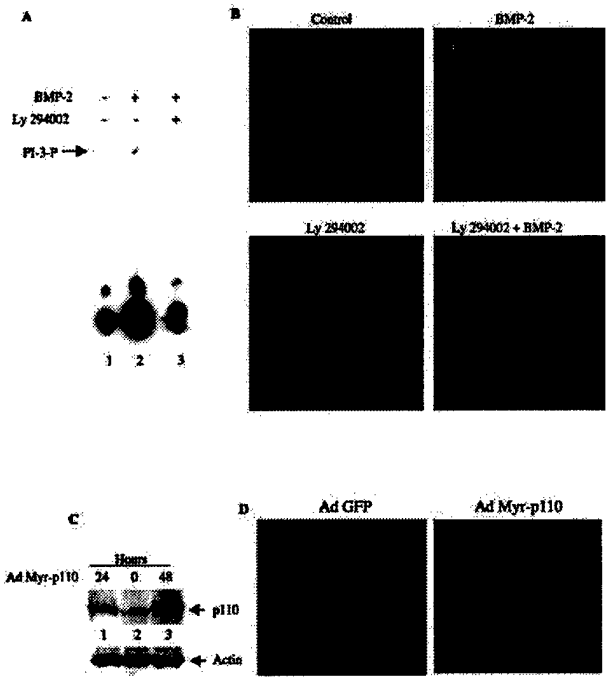

FIG. 2. **PI 3-kinase regulates expression of MHC in CL6 cardiomyocyte precursor cells.** *A*, PI 3-kinase inhibitor blocks BMP-2-induced PI 3-kinase activity. CL6 cells were incubated with 12.5 μM Ly294002 for 1 h before incubation with 100 ng/ml BMP-2 for 15 min. PI 3-kinase activity was determined in the anti-phosphotyrosine immunoprecipitates as described in Fig. 1. The *arrow* indicates the position of PI 3-phosphate. *B*, PI 3-kinase inhibitor prevents MHC expression. CL6 cells in eight-chamber slides were treated with Ly294002 followed by BMP-2. The cells were stained with MF-20 antibody as described under "Experimental Procedures." *C*, expression of constitutively active p110 subunit of PI 3-kinase. CL6 cells were infected with a multiplicity of infection of 50 for Ad Myr-p110 for the indicated periods of time (17, 20). 25 μg of cleared cell lysates was immunoblotted with anti-p110 antibody to detect the expression of the protein. The *bottom panel* shows immunoblot analysis of the same sample with actin antibody. *D*, expression of constitutively active PI 3-kinase increases MHC expression. CL6 cells were infected with Ad Myr-p110 or with a control virus Ad GFP as described (17, 20) After infection, the cells were grown in serum-deprived medium for 48 h. The cells were stained with MF-20 antibody as described under "Experimental Procedures."

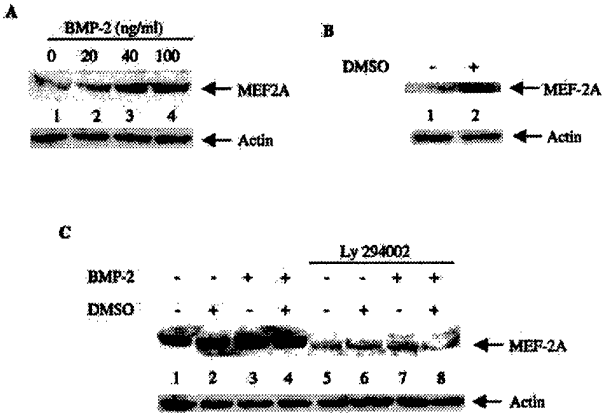

FIG. 3. **Effect of BMP-2 on MEF-2A protein expression in CL6 cells.** *A*, BMP-2 increases MEF-2A protein expression. Quiescent cells were incubated with different concentrations of BMP-2 for 24 h. Equal amounts of proteins were immunoblotted with the MEF-2A antibody. The *lower panel* shows immunoblot analysis of the same samples with anti-actin antibody to demonstrate equal loading. *B*, Me_2SO increases MEF-2A expression. Quiescent CL6 cells were incubated with 1% Me_2SO. Equal amounts of protein were immunoblotted with MEF-2A antibody. The *bottom panels* show the immunoblot analysis of the same samples with anti-actin antibody. *C*, inhibition of PI 3-kinase blocks MEF-2A expression. Quiescent CL6 cells were treated with Ly294002 for 1 h. Cells were then incubated either with BMP-2 or Me_2SO or BMP-2 plus Me_2SO. Equal amounts of protein were immunoblotted with anti-MEF-2A antibody. The *bottom panel* shows the immunoblot of the same samples with actin antibody to show equal loading.

catalytic subunit of PI 3-kinase. Infection of CL6 cells with this adenovirus vector showed expression of p110 within 24 h (Fig. 2*C*). To test the effect of PI 3-kinase on MHC expression, CL6 cells were infected with Ad Myr-p110. The cells were stained with MF-20. Expression of the constitutively active PI 3-kinase increased expression of sarcomeric MHC (Fig. 2*D*). These data indicate that PI 3-kinase is required for MHC-expressing mature cardiomyocyte formation in response to BMP-2.

PI 3-kinase Regulates BMP-2-induced MEF-2A Expression—Me_2SO-mediated cardiomyocyte differentiation of CL6 cells has previously been shown to induce MEF-2C expression (16). However, it is not known whether MEF-2A, which is also a marker for heart development, is expressed in CL6 cells (9). To address this and to examine the effect of BMP-2, CL6 cells were incubated with different concentrations of BMP-2. The lysates were immunoblotted with an antibody that recognizes MEF-2A. BMP-2 at a concentration of 40–100 ng/ml increased MEF-2A protein abundance (Fig. 3*A*). Me_2SO also induced MEF-2A expression in these cells (Fig. 3*B*).

To test the effect of PI 3-kinase on BMP-2-induced MEF-2A expression, we incubated CL6 cells with Ly294002 in the presence or absence of BMP-2. Cells were also treated with Me_2SO or Me_2SO plus Ly294002. As expected, BMP-2 and Me_2SO alone increased MEF-2A expression (Fig. 3*C*, compare *lanes 3* and *2* with *lane 1*). Addition of both BMP-2 and Me_2SO did not further increase the abundance of MEF-2A (Fig. 3*C*, *lane 4*). Inhibition of PI 3-kinase activity abolished both BMP-2-induced and Me_2SO-induced MEF-2A expression (Fig. 3*C*, compare *lane 7* with *3* and *lane 6* with *2*, respectively). These data indicate that PI 3-kinase regulates a common target, which modulates MEF-2A expression in CL6 cells in response to BMP-2 as well as Me_2SO.

PI 3-kinase Regulates BMP-2-induced MEF-2A DNA Binding—Because BMP-2 increased MEF-2A protein expression, we tested whether the growth and differentiation factor stimulates the DNA binding activity of the transcription factor. Nuclear extracts isolated from BMP-2-treated CL6 cells were incubated with a ^{32}P-labeled MEF-2 consensus oligonucleotide, and the products were analyzed by electrophoretic mobility shift assay. BMP-2 increased formation of protein·DNA complex (Fig. 4*A*, compare *lane 2* with *1*). Inclusion of a 100-fold excess of cold oligonucleotide completely prevented the protein·DNA complex formation, indicating the specificity of DNA-protein interaction (Fig. 4*A*, compare *lane 3* with *2*). To test the involvement of MEF-2A in the formation of protein·DNA complex, an antibody specific for MEF-2A was used in the EMSA. MEF-2A antibody inhibited the protein·DNA complex formation (Fig. 4*B*, compare *lane 3* with *2*). Note that the antibody partially supershifted the complex (Fig. 4*B*, *lane 3*, indicated by the *bracket*). A heterologous antibody against cyclin D1 did not have any effect on protein·DNA complex formation (Fig. 4*B*, *lane 4*). These data indicate that BMP-2 not only regulates the expression of MEF-2A in CL6 cells but also modulates its DNA binding activity to its cognate DNA element.

PI 3-kinase regulates receptor tyrosine kinase-induced transcription of target genes, which modulate cell survival (32). However, nothing is known about regulation of BMP-receptor serine threonine kinase-induced PI 3-kinase-dependent MEF-2 transcriptional activation. Therefore, to assess the functional

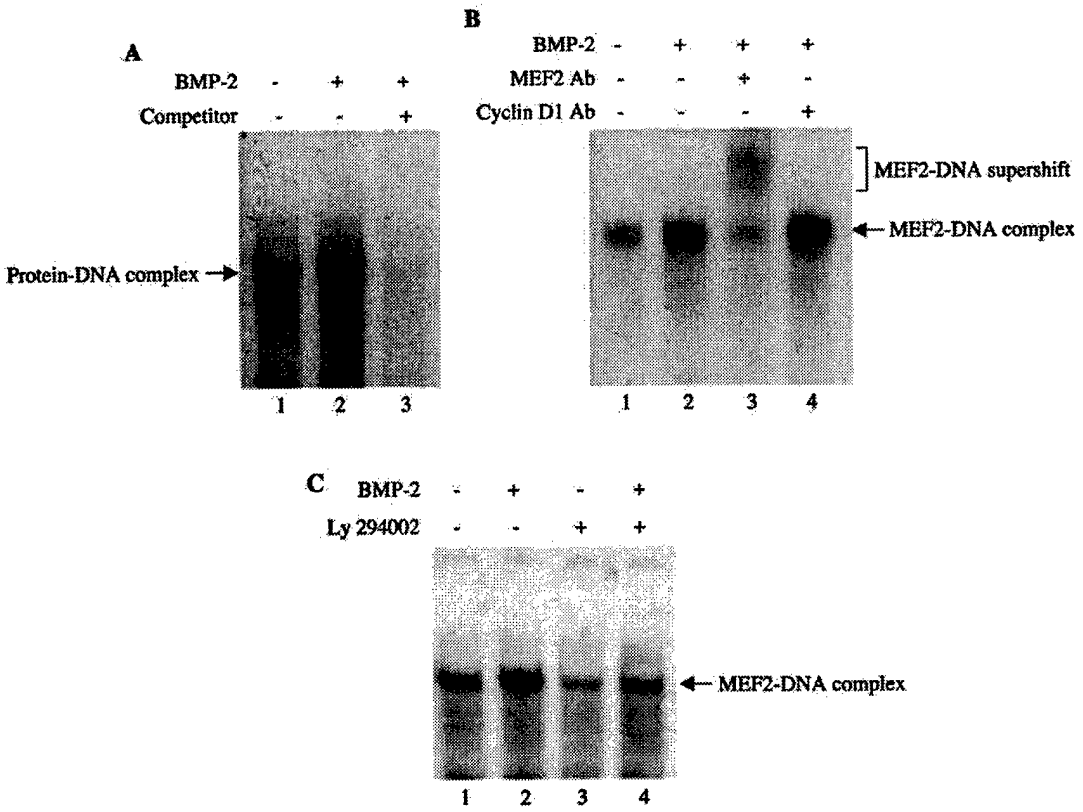

FIG. 4. **PI 3-kinase regulates BMP-2-induced MEF-2A DNA binding in CL6 cells.** *A*, BMP-2 increases MEF-2A DNA binding. Quiescent CL6 cells were incubated with BMP-2. Nuclear extracts (10 μg) were used in EMSA as described under "Experimental Procedures" (25, 27). For competition, 100-fold excess cold oligonucleotide was used with the nuclear extracts before the probe was added. The protein·DNA complex was separated by 5% polyacrylamide gel electrophoresis. The *arrow* indicates the specific protein·DNA complex. *B*, supershift analysis of BMP-2-induced protein·DNA complex. Nuclear extracts were incubated with MEF-2A antibody or nonspecific cyclin D1 antibody for 30 min on ice before incubation with the MEF-2A probe. Protein·DNA complexes were separated as described in *panel A*. The *arrow* indicates the protein·DNA complex. The *bracket* indicates the supershifted MEF-2A·DNA complex. *C*, inhibition of PI 3-kinase blocks BMP-2-induced MEF-2A DNA binding. CL6 cells were incubated with Ly294002 for 1 h before stimulation with BMP-2. Nuclear extracts were used in EMSA as described in *panel A*. The *arrow* indicates the protein·DNA complex.

consequences of BMP-2-induced PI 3-kinase activation in MEF-2A DNA binding, an electrophoretic mobility shift assay was carried out with nuclear extracts isolated from CL6 cells incubated with BMP-2 alone and BMP-2 plus Ly294002. Inhibition of PI 3-kinase activity by this pharmacological agent abolished BMP-2-induced MEF-2A·DNA complex formation (Fig. 4*C*, compare *lane 4* with *2*). These data indicate that activation of PI 3-kinase regulates BMP receptor serine threonine kinase-induced MEF-2-DNA interaction.

PI 3-kinase Regulates BMP-2-induced MEF-2-dependent Transcription—Transcriptional activation of MEF-2 family transcription factors is mediated by tyrosine kinase-dependent PI 3-kinase. For example, activation of insulin-like growth factor-1 receptor tyrosine kinase induces myogenic differentiation via PI 3-kinase-dependent increase in MEF-2 transcriptional activity (12). To directly examine the effect of PI 3-kinase on MEF-2-dependent transcription in BMP-2 receptor serine threonine kinase activation, we constructed a reporter plasmid in which four copies of the MEF-2 DNA binding element were cloned upstream of the SV-40 basal promoter driving luciferase cDNA (Fig. 5*A*). The reporter plasmid was transfected into CL6 cells. Incubation of these transiently transfected cells with BMP-2 increased transcription of the reporter gene, indicating that BMP-2 stimulates transcription from the MEF-2 DNA element (Fig. 5*B*). Furthermore, cotransfection of MEF-2A cDNA with the reporter plasmid resulted in a significant increase in the transcriptional activity demonstrating that the reporter construct is responsive to the MEF-2A transcription factor (Fig. 5*B*). Addition of BMP-2 to these cotransfected cells showed no further significant additive effect. To test the involvement of PI 3-kinase in the BMP-2-induced transcription of this reporter gene, transiently transfected cells were incubated with the PI 3-kinase inhibitor, Ly294002, before addition of BMP-2. Inhibition of PI 3-kinase significantly blocked BMP-2-induced transcription of the reporter gene driven by the MEF-2 DNA element (Fig. 5*C*). To confirm this observation, reporter construct was cotransfected with a dominant negative 85-kDa subunit of PI 3-kinase. Transiently transfected cells were incubated with BMP-2. Dominant negative PI 3-kinase significantly prevented BMP-2-induced transcription of the reporter gene (Fig. 5*D*). These data indicate that BMP-2-induced PI 3-kinase activity is necessary for transcriptional activation of MEF-2.

MEF-2A Regulates BMP-2 Gene Transcription in a PI 3-kinase-dependent Manner—We have shown previously that BMP-2 autoregulates its own transcription in osteogenic cells (29, 30). To investigate the involvement of PI 3-kinase in BMP-2 transcription, we used a reporter construct in which the firefly luciferase gene is driven by the promoter of the BMP-2 gene (BMP-2-LUC) (29). This plasmid was transfected either with vector alone or dominant negative p85 subunit of PI 3-kinase into CL6 cells. Incubation of transiently transfected cells with

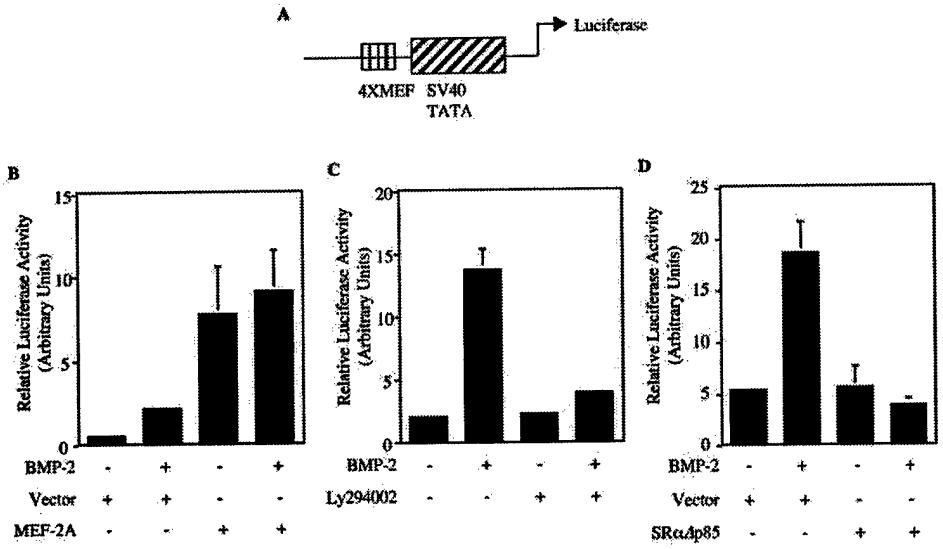

FIG. 5. **PI 3-kinase regulates MEF-2A transcriptional activity.** *A*, schematic showing the structure of the reporter plasmid in which luciferase gene is driven by four copies of MEF-2 DNA consensus element (24) from SV-40 basal promoter. *B*, BMP-2 and MEF-2A regulate reporter transcription. The reporter construct in *panel A* was cotransfected with vector or MEF-2A expression plasmid into CL6 cells. A *Renilla* luciferase plasmid was included with each transfection mix. Transiently transfected cells were incubated with BMP-2. Cell lysates were used for luciferase activity as described under "Experimental Procedures." *C*, PI 3-kinase regulates BMP-2-induced reporter gene transcription. The same reporter plasmid in *panel A* was cotransfected with the *Renilla* plasmid into CL6 cells. The transiently transfected cells were treated with Ly294002 for 1 h before addition of BMP-2. Cell lysates were assayed for luciferase activity as described under "Experimental Procedures." *D*, dominant negative PI 3-kinase inhibits BMP-2-induced reporter gene transcription. The reporter plasmid in *panel A* was cotransfected with vector or pSRαΔp85 coding for dominant negative p85 subunit of PI 3-kinase. The cells were treated with BMP-2, and the lysates were assayed for luciferase activity as described under "Experimental Procedures."

BMP-2 increased the reporter gene expression, indicating that BMP-2 autoregulates its own transcription in these cells similar to autoregulation in osteoblasts (Fig. 6*A*) (28, 29). However, transfection of dominant negative PI 3-kinase completely blocked BMP-2-induced BMP-2 transcription (Fig. 6*A*). These data indicate that, in CL6 cardiomyocyte precursor cells, PI 3-kinase regulates BMP-2 gene transcription induced by BMP-2.

To investigate the role of MEF-2 family of transcription factors in regulating the transcription of BMP-2, we analyzed the 5′-flanking sequence of BMP-2 gene. This analysis revealed the presence of three MEF-2 elements (30). To examine involvement of MEF-2A in BMP-2 transcription, we transfected the BMP-2-LUC reporter plasmid along with the MEF-2A expression vector into CL6 cells. Ectopic expression of MEF-2A increased BMP-2 promoter activity to an extent similar to that induced by BMP-2 alone (Fig. 6*B*), suggesting that MEF-2A regulates BMP-2 gene transcription in CL6 cells. Next, we examined the involvement of PI 3-kinase in the regulation of the MEF-2A-dependent transcription of BMP-2 gene. BMP-2-LUC was cotransfected with the MEF-2A expression vector alone or along with dominant negative PI 3-kinase. As expected, MEF-2A increased transcription of the BMP-2 gene (Fig. 6*C*). Expression of dominant negative PI 3-kinase completely blocked the MEF-2A-dependent BMP-2 transcription (Fig. 6*C*). Together these data indicate that BMP-2-induced PI 3-kinase may regulate MEF-2A transcriptional activity, which in turn regulates BMP-2 gene transcription.

MEF-2A Induces Formation of Mature Cardiomyocyte-expressing MHC—Because MEF-2A regulates BMP-2 expression and BMP-2 is necessary for differentiation of CL6 cells to form mature cardiomyocytes (16), we examined the effect of MEF-2A expression on this process. CL6 cells were transfected with MEF-2A expression vector. The transfected cells were stained with the MF-20 antibody for sarcomeric MHC, which is expressed in mature cardiomyocyte. Expression of MEF-2A significantly increased expression of MHC in cardiomyocytes (Fig. 7, compare *panel B* with *A*). To address the involvement of PI 3-kinase, we treated the MEF-2A-transfected cells with Ly294002. The cells were then stained with MF-20 antibody. Inhibition of PI 3-kinase by the pharmacological inhibitor significantly blocked MEF-2A-induced MHC expression (Fig. 7, compare *panel D* with *B*). These data demonstrate that PI 3-kinase regulates MEF-2A-induced cardiomyocyte differentiation of CL6 cells.

DISCUSSION

The present study shows that, in cardiomyocyte precursor cells, BMP-2 activates PI 3-kinase. We demonstrate that PI 3-kinase regulates differentiation of precardiac cells to MHC-expressing mature cardiomyocytes. We show that BMP-2-induced expression of MEF-2A transcription factor, its DNA binding, and transcriptional activity requires activation of PI 3-kinase. Also we demonstrate that PI 3-kinase modulates autoregulation of BMP-2 gene transcription in these cells. Furthermore, we provide the first evidence that MEF-2A regulates transcription of BMP-2 gene and that this MEF-2A-dependent transcriptional regulation is PI 3-kinase-sensitive.

The role of PI 3-kinase in growth factor and cytokine-induced signal transduction where receptor and nonreceptor tyrosine kinases are involved is well established (31). PI 3-kinase regulates many biological activities, including proliferation, migration, endocytosis, protein transport, and secretion (33). A role for PI 3-kinase has recently been shown in adipocyte, osteogenic, and myogenic differentiation (11, 17, 34–36). BMP-2 is known to play important roles in cardiac development (6). However, the molecular basis for its effect on the differentiation process is poorly understood. We have shown here that BMP-2, which induces differentiation of precursor cells to mature cardiomyocytes, increases PI 3-kinase activity in these cells (Fig. 1*A*).

PI 3-kinase consists of a catalytic p110 subunit and a regu-

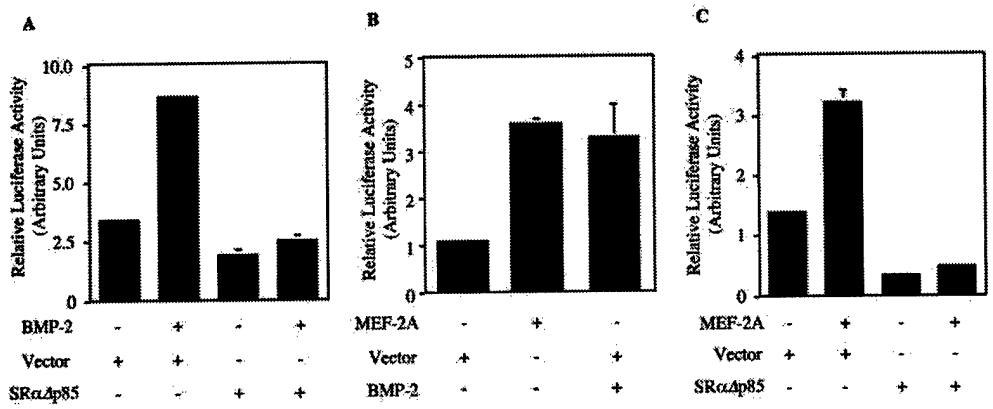

FIG. 6. **PI 3-kinase regulates MEF-2A-dependent BMP-2 transcription.** *A*, dominant negative PI 3-kinase inhibits BMP-2-induced BMP-2 transcription in CL6 cells. BMP-2-LUC reporter plasmid was cotransfected into CL6 cells with either vector or pSRαΔp85, which encodes dominant negative p85 subunit of PI 3-kinase. The cells were incubated with BMP-2. Lysates were assayed for luciferase activity as described under "Experimental Procedures." *B*, expression of MEF-2A stimulates BMP-2 transcription. CL6 cells were cotransfected with BMP-2-LUC reporter plasmid and MEF-2A expression vector. Also vector-transfected cells were incubated with BMP-2. Cell lysates were used for luciferase assay as described under "Experimental Procedures." *C*, dominant negative PI 3-kinase blocks MEF-2A-mediated BMP-2 transcription. BMP-2-LUC reporter plasmid was cotransfected with MEF-2A expression vector and pSRαΔp85. Cell lysates were used for luciferase activity as described under "Experimental Procedures."

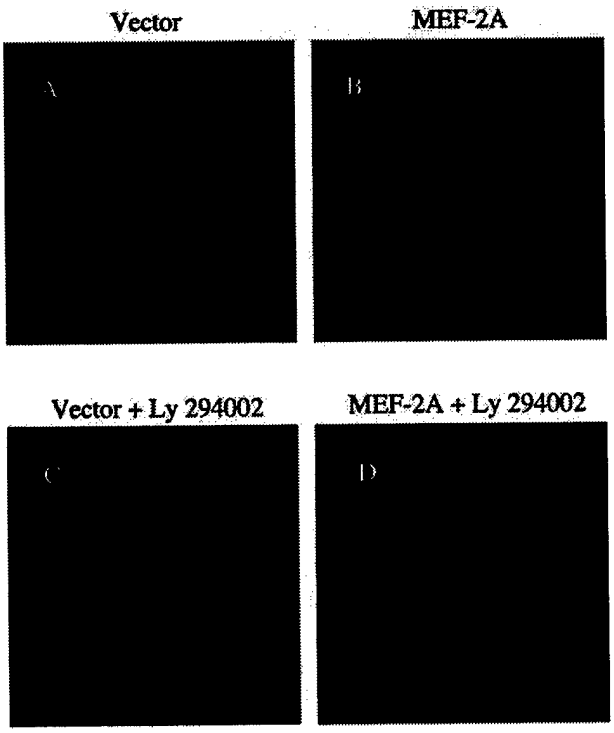

FIG. 7. **MEF-2A regulates MHC expression.** CL6 cells were transfected with the vector or MEF-2A expression plasmid (*A* and *B*). At 4 h post-transfection, the cells were incubated with Ly294002 (*C* and *D*). After 48 h, the cells were stained with MF-20 antibody as described under "Experimental Procedures."

latory SH-2 domain-containing p85 subunit (31). One mechanism of tyrosine kinase-induced activation of this lipid kinase depends upon its association via its SH-2 domain to the phosphotyrosine residue of the tyrosine-phosphorylated proteins, which include the tyrosine kinase itself and its substrates (31). This mechanism demonstrates direct physical association of PI 3-kinase activity with the tyrosine-phosphorylated protein fraction. Indeed, BMP-2-stimulated PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates suggests the presence of PI 3-kinase in a tyrosine-phosphorylated signaling complex (Fig. 1*B*). Because BMP receptors possess serine threonine kinase activity and do not undergo tyrosine phosphorylation themselves, it remains to be identified with which tyrosine-phosphorylated protein PI 3-kinase is associated upon BMP-2 activation of CL6 cells.

BMP-2 is necessary for cardiomyocyte development *in vivo* and *in vitro* (5, 6, 16). Sarcomeric MHC is expressed in mature cardiomyocyte. It is established that BMP-2 increases MHC expression during cardiomyocyte differentiation (16, Fig. 2*B*). However, the signal transduction mechanism by which MHC is expressed in mature cardiomyocytes is not precisely known. We demonstrate that PI 3-kinase regulates BMP-2-induced MHC expression during cardiomyocyte differentiation (Fig. 2, *B* and *D*).

BMP-2 exerts its biological effect by receptor-mediated serine phosphorylation of BMP-specific Smad 1, Smad 5, and Smad 8 (1). After phosphorylation, the Smad proteins heterodimerize with Smad 4 to translocate to the nucleus and directly stimulates transcription of target genes necessary for biological activity. Thus ectopic expression of Smad 1 has been shown to promote survival of cardiac myocytes (15). BMP-2 also stimulates expression of cardiac-specific genes such as MEF-2A during cardiomyocyte differentiation (9). Homozygous mutation in MEF-2C, a member of the MEF-2 family of transcription factor, is embryonically lethal due to defect in cardiac development (37). The phenotype is very similar to that seen in BMP-2 null mice (6). During mouse embryogenesis, MEF-2C is expressed in the precardiogenic mesoderm at embryonic day E7.75, whereas MEF2A is expressed beginning at E8.25 (38, 39). BMP-2 is detectable as early as E7.5 (6), indicating that this growth and differentiation factor could play a role in expression of MEF-2A. Here we show that, in CL6 cardiomyocyte precursor cells, BMP-2 stimulated expression of MEF-2A (Fig. 3*A*). These data correlate with the expression pattern of BMP-2 and MEF-2A and a role of BMP-2 in MEF-2A expression during mouse cardiac development (6, 38, 39).

The signaling mechanism regulating the expression of MEF-2A transcription factor is largely unknown. Recently, PI 3-kinase has been shown to regulate the transcriptional activity of MEF-2 in myogenic cells (40). However, PI 3-kinase does not have any effect on expression of MEF-2A (12). In contrast to this observation, we demonstrate that BMP-2-induced expression of MEF-2A is regulated by PI 3-kinase in CL6 cardiomyocyte precursor cells (Fig. 3*C*).

PI 3-kinase has been shown to regulate insulin-like growth factor-1-induced myogenic differentiation (34, 36, 40). Expression of constitutively active PI 3-kinase increased transcription of myogenin (40). This increase in myogenin transcription was ascribed to the increase in MEF-2-mediated transcriptional activity (40). In another study, Tamir and Bengal (12) showed that PI 3-kinase regulates the transcriptional activity of MEF-2 without having any effect on its DNA binding property. In contrast, in CL6 cardiomyocyte precursor cells, we show that BMP-2 increases MEF-2A·DNA interaction, and inhibition of PI 3-kinase activity blocks this DNA binding, resulting in significant reduction in MEF-2-dependent transcription of a reporter gene (Figs. 4 and 5). The reduction in protein·DNA complex formation and transcription from the MEF-2 DNA element may be in part due to the decreased expression of MEF-2A we observed in the presence of PI 3-kinase inhibition (Fig. 3). On the other hand, PI 3-kinase has been shown to increase the phosphorylation of MEF-2A, which regulates the transcriptional activity of this protein (12). Therefore, we cannot completely rule out the possibility of post-translational modification of MEF-2A in regulating its DNA binding, resulting in its transcriptional activation in response to BMP-2 in CL6 cells.

We have shown previously that BMP-2 autoregulates its expression during osteogenesis (29). This is one of the mechanisms by which BMP-2 maintains its sustained effect during osteoblast differentiation. Expression of the BMP-2 ortholog *dpp* is also regulated by *dpp* signaling during retinal differentiation in *Drosophila* (41). Here we have shown BMP-2 autoregulates also its own transcription in cardiomyocyte precursor cells (Fig. 6*A*). Others have shown that, during cardiac development in mouse, BMP-2 and MEF-2A are expressed in precardial mesoderm, thereby suggesting a possible role of each protein in the other's expression (6, 38, 39). In fact, we have shown that BMP-2 stimulates MEF-2A expression in CL6 cells (Fig. 3). Analysis of the BMP-2 5′-flanking sequence revealed the presence of an MEF-2 consensus DNA element in the BMP-2 promoter (30). The observation that expression of exogenous MEF-2A increased the transcription from BMP-2 promoter indicates that the DNA elements present in the BMP-2 5′-flanking sequence are responsive to this transcription factor (Fig. 6*B*). How this transcriptional activation is mediated is not known at this point. However, it is known that activation of the BMP receptor increases the transcriptional activity of the BMP-specific Smad proteins. Interaction between transforming growth factor β-specific Smad 2 and MEF-2A has been described in C2C12 myoblasts (42). In fact this interaction significantly increased the transcription of the target reporter gene, indicating the relevance of the physical association of MEF-2A and Smad 2. Therefore, our observation that MEF-2A significantly increased BMP-2 transcription may represent the presence of cross-talk between BMP-specific Smad(s) and MEF-2A or direct interaction of these transcription factors.

The cystine knot protein noggin binds BMPs in the two hydrophobic patches, which directly interact with the BMP receptor and block BMP signal transduction (43–45). Thus noggin prevents cardiac gene expression, including MEF-2A in chick embryo explant (9). Similar to the precardiac parental cells, precardiac cells expressing noggin do not undergo cardiac differentiation in response to Me_2SO, indicating that sustained action of BMP-2 is necessary for this process (16, 46). We also show that CL6 cells expressing noggin are incapable of activating MEF-2A in response to BMP-2 (Supplemental Fig. S1).

Because BMP-2 stimulates MEFF-2A expression (Fig. 3*A*) and MEF-2A increases transcription of BMP-2 gene (Fig. 6), one possible explanation of this observation may be that BMP-2-mediated expression of MEF-2A may maintain the level of BMP-2 via its increased transcription. This pathway may represent one of the mechanisms of BMP-2 autoregulation, which maintains the level of BMP-2 required for cardiomyocyte differentiation (Fig. 8). Furthermore our data show that ectopic expression of MEF-2A increased expression of MHC (Fig. 7). Whether this effect of MEF-2A is due to its effect on BMP-2 expression, which in turn induces cardiomyocyte differentiation, is yet to be established. However, we demonstrate that MEF-2A-dependent MHC expression is PI 3-kinase-sensitive (Fig. 7).

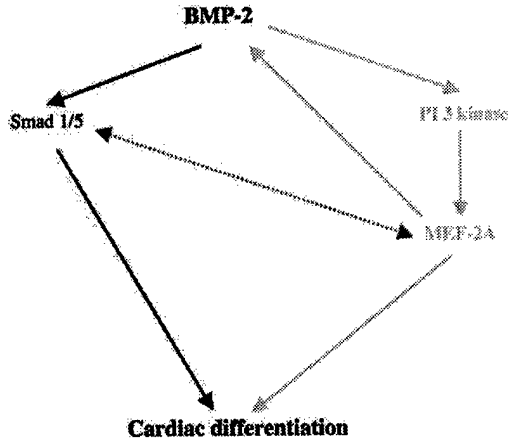

FIG. 8. **Scheme of BMP-2-induced MEF-2A-dependent BMP-2 expression and cardiac differentiation.** It is established (5, 6) that BMP-2 stimulates BMP-specific Smads to regulate cardiac differentiation (indicated by *black arrows*). We demonstrate that BMP-2 increases PI 3-kinase activity, which regulates MEF-2A-dependent transcription of BMP-2 suggesting a plausible mechanism of autoregulation of BMP-2 expression. Also, increased MEF-2A expression regulates MHC expression, a marker for cardiac differentiation (*green arrows*). The *dotted red arrow* indicates a plausible interaction between BMP-specific Smad and MEF-2A (yet to be identified). This notion is based on the observation that Smad 2, a transforming growth factorβ-specific Smad, has been shown to interact directly with MEF-2A (42).

In the present study, we provide the evidence that PI 3-kinase modulates BMP-2 gene transcription in CL6 cells (Fig. 6*A*). Several downstream targets of PI 3-kinase have been identified (47, 48). One is the Akt serine threonine kinase, which has been shown to play important roles in myogenic, osteogenic, and adipose differentiation (11, 17, 34–36). Other targets of PI 3-kinase are the novel isoforms of PKC (49). PKCε and PKCδ have recently been shown to induce phosphorylation of MEF-2A, resulting in increased MEF-2A transcriptional activity (50). In cardiomyocytes, the target of PI 3-kinase that modulates MEF-2A function has not yet been identified.

In summary, we have shown that BMP-2 regulates expression of MHC in cardiomyocytes in a PI 3-kinase-sensitive manner. We have also shown that BMP-2 stimulates expression of MEF-2A transcription factor. Our data demonstrate that PI 3-kinase regulates its expression, DNA recognition, and transcriptional activity. Finally, we have provided the first evidence that BMP-2-induced BMP-2 transcription is mediated in a PI 3-kinase-sensitive and MEF-2-dependent manner. Taken together our data indicate that BMP-2 and MEF-2A orchestrate cardiac development in a concerted way using the PI 3-kinase signal transduction pathway.

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